Cytokines and Chemokines in the Pathogenesis of Low Back Pain

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Dedication

For Mum & Dad

Abstract

Degeneration of the intervertebral disc (IVD) is thought to account for 40% of all cases of chronic low back pain. Alterations in the behaviour of the IVDs' native cell population mediate the processes that lead to structural failure, as seen in IVD degeneration.

Cytokines are implicated in this process, several studies have identified that Interleukin-1 (IL-1) and Tumour Necrosis Factor-α (TNF-α) expression is increased in degenerate IVDs compared to their normal counterparts. Furthermore, it has been shown *in vitro* that these cytokines stimulate alterations in the behaviour of the IVDs' native cells in a similar manner to those observed in IVD degeneration. However, IL-1 and TNF-α are only two of a large group of intercellular signalling molecules known as cytokines, and studies investigating the expression of other cytokines in the human IVD are limited.

This thesis demonstrates the production of numerous cytokines and chemokines (chemoattractant cytokines) by the human IVDs' native cell population. Detailed gene and protein expression studies identified several novel cytokines and chemokines that are differentially expressed in cells isolated from degenerate or prolapsed IVDs compared to those isolated from the normal counterpart. Co-expression of receptors for these molecules was also identified, indicating a capacity of these cells to respond to cytokine intercellular signalling.

The response of IVD cells to cytokine and chemokine stimulation *in vitro* was investigated. The data presented indicates that inter-regulatory relationships exist between the cytokines and chemokines of the intervertebral disc. Particularly, IL-1 exerts modulatory potential over the expression of other cytokines and chemokines by IVD cells. Effects of stimulation were also observed in relation to reduced anabolic metabolism and increased catabolic metabolism, both of which are characteristic features of IVD degeneration.

Together, the findings presented in this thesis indicate that cytokines and chemokines are integral to the pathogenesis of IVD degeneration and prolapse that may lead to low back pain.

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Abbreviations

18S	Eukaryotic 18S rRNA
ACTB	β-Actin
ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motif
AF	Annulus Fibrosus
AR	Antigen Retrieval
BSA	Bovine Serum Albumin
Caspase	Cysteinyl-Aspartate-Specific Protease
CD4	Cluster of Differentiation 4
cDNA	Complementary DNA
CEP	Cartilaginous End Plate
CSF	Colony Stimulating Factor
CSF R	Colony Stimulating Factor Receptor
C-terminal	Carboxylic Acid Terminal
DAB	3-3'-Diaminobenzidine Tetrahydrochloride
DAG	Diacylglycerol
DE	Directly Extracted
DMEM	Dulbecco's Modified Eagles Media
DNA	Deoxyribonucleic Acid
DR	Death Receptor
ECM	Extracellular Matrix
EDTA	Ethylene Diaminetra-Acetic Acid
FasL	Fas Ligand
FFPE	Formalin-Fixed Paraffin Embedded
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Guanosine Diphosphate
gp130	Interleukin-6 Family Signal Transducing Protein

GTP	Guanosine Triphosphate
HCl	Hydrochloric Acid
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HRP	Horseradish Peroxidase
ICE (a.k.a. Caspase-1)	Interleukin-1 Converting Enzyme
IFN	Interferon
IFN R	Interferon Receptor
IgG	Immunoglobulin
IHC	Immunohistochemistry
IKK	IκB Kinase
IL	Interleukin
IL R	Interleukin Receptor
IL-1Ra	Interleukin-1 Receptor Antagonist
IL-1RAcP	Interleukin-1 Receptor Accessory Protein
IMS	Industrial Methylated Spirit
IP3	Inositol-1,4,5 Triphosphate
LBP	Low Back Pain
LDA	Low Density Array
LIF	Leukaemia Inhibitory Factor
LIFR	Leukaemia Inhibitory Factor Receptor
MAPK	Mitogen Activated Protein Kinase
MMLV	Moloney Murine Leukaemia Virus
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear Factor-κB
NGF	Nerve Growth Factor
NP	Nucleus Pulposus
N-terminal	Amino Terminal
OA	Osteoarthritis
OSM	Oncostatin-M

OSMR	Oncostatin-M Receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK1	3-Phosphoinositide-Dependent Protein Kinase 1
PGK1	Phosphoglycerate Kinase 1
PI3K	Phosphatidyl-Inositol-3 Kinase
PKC	Protein Kinase C
PLC	Phosphoinositide-Specific Phospholipase C
PM	Post-Mortem
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT	Reverse Transcription
SFM	Serum Free Media
TACE (a.k.a. ADAM17)	Tumour Necrosis Factor Converting Enzyme
TAK1	TGF-β Activated Kinase
TBS	Tris-Buffered Saline
TGF-β	Transforming Growth Factor-β
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor Receptor
TRAF	TNF Receptor Associated Factor
VB	Vertebral Body

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1 General Introduction

1.1 The Spine

The human spine, or vertebral column, is comprised of 26 bones: 24 vertebrae, the sacrum and the coccyx. It functions to provide a column of support to the body and to transfer weight through to the appendicular skeleton of the lower limbs (Martini & Ober, 2005).

The vertebral column can be divided into 5 anatomical regions; cervical, thoracic, lumbar, sacral and coccygeal (superior to inferior). Each vertebra comprises of a vertebral body, vertebral arch and articular processes. It is the vertebral body that is responsible for the transfer of weight along the axis of the vertebral column (Martini & Ober, 2005). The bodies of adjacent vertebra are separated by an intervertebral disc (IVD) (Figure 1-1).

1.1.1 The Lumbar Spine

The lumbar spine comprises 5 vertebra (L1 – L5) and 5 IVDs separating the vertebral bodies and between vertebra L5 and the sacrum, S1 (Figure 1-1). The structure of the lumbar spine is stabilised by encasing longitudinal ligaments (anterior, posterior and posterio-lateral) and the musculature of the lower back.

1.2 The Intervertebral Disc

The IVD is a fibro-cartilaginous structure that separates adjacent vertebra of the spine (Peacock, 1952) (Figure 1-1). Each IVD is an amphiarthrosis (Taylor *et al.*, 1992), or cartilaginous joint, and as such permits a small degree of flexion, extension and lateral bending between adjacent vertebrae. Summated the small movement at each IVD constitutes the mobility of the spine.

IVDs function to maintain the correct biomechanical separation of adjacent vertebra (Adams, 2002). They are resistant to compressive load and maintain separation even throughout weight bearing (Lu *et al.*, 1996). IVDs are comprised of three distinct tissue regions; the end plates, the annulus fibrosus (AF) and the nucleus pulposus (NP) (Peacock, 1952).

1.2.1 The End Plates

In the adult vertebral column, opposing vertebral bodies are covered by the end plates (Peacock, 1952). These are a layer of hyaline cartilage (cartilaginous end plate, CEP) and the central parts of the superior and inferior cortical bone surfaces (bony end plate) of the vertebral bodies that they cover (Moore, 2006).

The structure of the CEP is seen to vary across the IVD, with tissue resembling that of which it is most closely associated (Roberts *et al.*, 1989a). The central region is thinnest, approximately 0.1mm and being NP associated, is more gelatinous than the peripheral fibrous AF associated regions, that increase to approximately 1.6mm in thickness (Roberts *et al.*, 1989b). The CEP confers the structural resilience that

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Figure 1-1 Schematic Representation of the Anatomy of the Spine

(A) The intervertebral disc (IVD) functions to permit flexion throughout the spine. Each IVD and adjacent two vertebra form a 'motion segment'. The distance between adjacent vertebral bodies is termed 'IVD height' (black arrow). The lumbar spine comprises 5 vertebra (L1 – L5) and 5 IVDs separating the vertebral bodies and between vertebra L5 and the sacrum, S1. (B) Each IVD comprises of an outer fibrous annulus fibrosus and inner gelatinous nucleus pulposus. Posterior to the axis of the vertebral bodies and IVDs are the vertebral arches. These are bony processes that extend from each vertebra, they encase and protect the spinal canal through which the spinal cord runs. Figures adapted from Chen & Zieve, (2008) and Painter, (2009).

prevents loads transferred through the IVD from fracturing the bone of vertebral bodies (Moore, 2006).

1.2.2 The Annulus Fibrosus

The AF constrains the NP around the periphery and binds together the outer rims of adjacent vertebrae (Peacock, 1952). It can be subdivided into morphologically distinct outer and inner regions;

1.2.2.1 The Outer Annulus Fibrosus

The outer AF consists primarily of type I collagen fibres arranged into a series of concentric lamellae (Eyre & Muir, 1976). The collagen fibres are inserted into the cortical bone at the vertebral body rim (Hilton & Ball, 1984) and pass obliquely between vertebral bodies (Eyre & Muir, 1976) (Figure 1-2). The orientation of fibres is reversed in successive lamellae and this arrangement confers concentric tissue elasticity (Walmsley, 1953; Adams, 2002).

1.2.2.2 The Inner Annulus Fibrosus

The inner AF is the transition zone between the organised fibrous structure of the outer AF and the irregular gelatinous structure of the NP (Walmsley, 1953; Baer *et al.*, 2001). A smooth transition is seen from type I to type II collagen fibres from outer to inner regions with increasing interspersion of hydrating proteoglycan molecules towards the inner (Eyre & Muir, 1976).

The AF confers the structural resilience to prevent excessive separation of the vertebra during flexion and withstands internal pressure as a response to loading, to resist deformity and prolapse of the IVD (Prescher, 1998).

1.2.3 The Nucleus Pulposus

The NP is the central component of the IVD, it is constrained above and below by the end plates and around the periphery by the AF (Peacock, 1952). NP is a highly hydrated gelatinous tissue containing large amounts of aggregating proteoglycans (Stevens *et al.*, 1979; Taylor *et al.*, 1992) held together loosely by a network of type II collagen fibres (Stevens *et al.*, 1979).

The proteoglycan content renders the structure hydrophilic and so it imbibes water (Urban & McMullin, 1988). This generates a swelling pressure within the IVD sufficient to force apart, and maintain separation of, adjacent vertebral bodies (Urban & McMullin, 1988; Mwale *et al.*, 2004).

1.2.4 Vasculature

With the exception of the outermost AF the adult human IVD is avascular (Peacock, 1952; Hassler, 1969; Rudert & Tillmann, 1993). Blood supply to the adjacent vertebral bodies is by exterior vertebral arteries that encircle the ‘waist’ of each vertebra (Crock, 1993). From these, centrum branches penetrate to form a grid

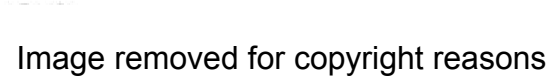


Figure 1-2 Schematic Representation of the Structure of the Outer Annulus Fibrosus

The outer annulus fibrosus of the human lumbar IVD is formed from approximately 20 concentric lamellae. Each lamella contains approximately 40 collagen fibre bundles that pass obliquely between the superior and inferior vertebral bodies. The arrangement of fibres in successive lamellae is reversed, α = approximately 30°. Figure adapted from Adams & Roughley, (2006).

within the vertebral body, off which vertically oriented tributaries pass up- and down-wards toward the end plates (Hassler, 1969; Crock, 1993). These give rise to capillary circuits in the region of the CEP (Crock, 1993; Rudert & Tillmann, 1993).

1.2.5 Nutrition

Nutritional support to the IVD is provided by diffusion across the CEP from the capillary networks of the superior and inferior vertebral bodies (Urban *et al.*, 2004) and from the capillary networks of the outer AF (Urban *et al.*, 2004) (Figure 1-3). Similarly, metabolic waste is excreted by this route (Holm *et al.*, 1981).

1.2.6 Innervation

The posterior longitudinal ligament is innervated by the lumbar sinuvertebral nerves (Bogduk *et al.*, 1981), fibres from these are seen to form free nerve endings in the adjacent posterior AF (Bogduk *et al.*, 1981). The anterior and lateral aspects of the IVD are supplied by branches from the *rami communicantes* (Bogduk *et al.*, 1981). In either case, nerve fibres are not seen to penetrate further than the outermost one third of the AF (Bogduk *et al.*, 1981).

1.2.7 Development

During embryonic organogenesis, the NP is formed from the notochord (Walmsley, 1953) whereby notochordal cells secrete a 'mucoid substance' that will later become the NP extracellular matrix (ECM) (Walmsley, 1953). This is surrounded by the inner AF, formed from the embryonic pre-cartilage structure – the perinotochordal mesenchyme (Walmsley, 1953; Rufai *et al.*, 1995), and the outer AF that is derived from surrounding fibrous mesenchymal tissue (Walmsley, 1953; Rufai *et al.*, 1995; Aulisa *et al.*, 1998).

After 6 month's gestation a decline in the notochordal cell population is seen and 'chondrocyte like' cells begin to appear in the NP (Walmsley, 1953). These are thought have migrated from the pre-cartilage structure of the inner AF and contribute collagen fibres to the mucoid NP (Walmsley, 1953).

Organisation of IVD tissue structures at full-term is as of that of an adult, however increased vasculature is observed at birth compared to that in mature IVDs (Walmsley, 1953; Crock, 1993). Immediately post-natally, a sharp decrease in IVD cell numbers occurs (Liebscher *et al.*, 2011) which corresponds with decreasing vasculature supply into the IVD (Crock, 1993). Cell numbers decline as a result of notochordal cell death, leaving only 'chondrocyte like' cells within the NP (Walmsley, 1953).

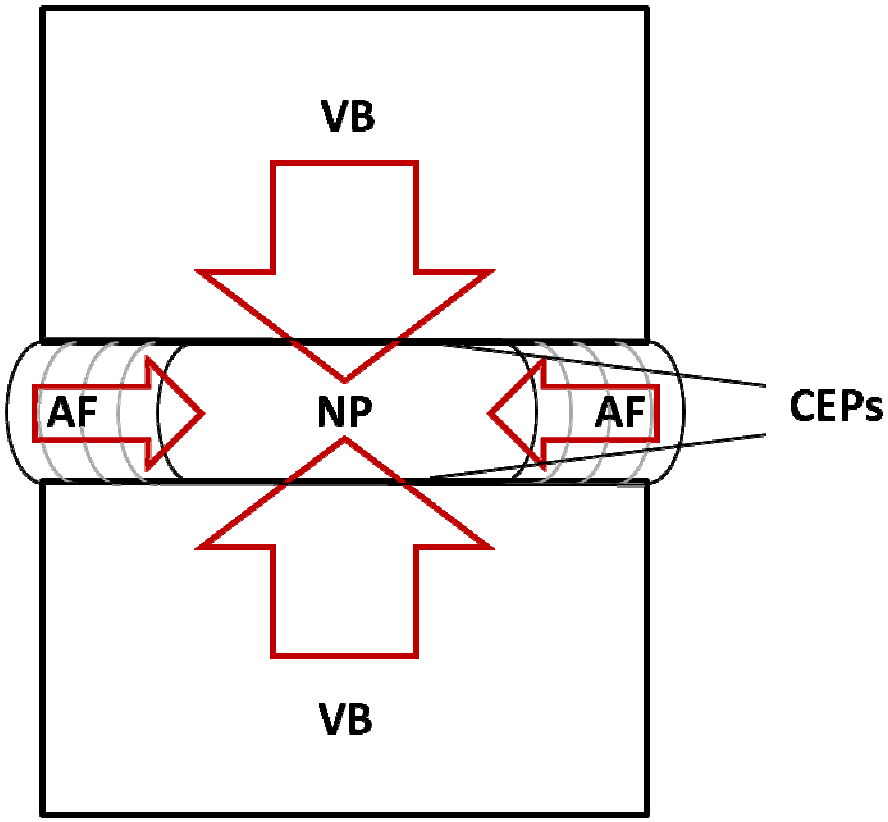


Figure 1-3 Schematic Representation of Diffusion Routes into the Intervertebral Disc

Nutrition is provided to the central nucleus pulposus (NP) region of the intervertebral disc by diffusion across the cartilaginous end plates (CEPs) and through the annulus fibrosus (AF) (red arrows). Capillary networks exist in the subchondral bone of the vertebral bodies (VB) immediately adjacent to CEPs and in the outer third of the AF.

1.2.8 The Cells of the Intervertebral Disc

The adult human IVD contains three native cell populations, one in each tissue region and each exhibiting morphological distinction from the others;

1.2.8.1 Cartilaginous End Plate Cells

The CEP is home to a population of chondrocytes (Moore, 2006). This region exhibits the highest cell density of any IVD tissue with the central region of the young adult human lumbar CEP containing ~18,000 cells/mm^3 (Liebscher *et al.*, 2011).

1.2.8.2 Annulus Fibrosus Cells

Native AF cells are flattened, elongated, bipolar and aligned parallel to tissue collagen fibres (Walmsley, 1953; Chelberg *et al.*, 1995). This morphology has resulted in them being termed 'fibroblast like'. Throughout the AF there is a transition from the flattened morphology observed in the outermost region where cells are characterised by type I collagen expression (Chelberg *et al.*, 1995), to a more rounded morphology in the innermost where cells are characterised by both type I and II collagen expression (Chelberg *et al.*, 1995). The young adult human lumbar AF cell population is sparse, ~2,600 cells/mm^3 (Liebscher *et al.*, 2011). Individual cells are isolated, residing in distinct lacuna within the ECM, and separated from any direct cell-cell contact with neighbours.

1.2.8.3 Nucleus Pulposus Cells

Native NP cells have a rounded morphology and appear microscopically very similar to the chondrocytes of the CEP (Walmsley, 1953). This morphology has resulted in them being termed 'chondrocyte like' and cells from this region are characterised by expression of type II collagen (Chelberg *et al.*, 1995). As in the AF the cell population is sparse, ~2,600 cells/mm^3 in the young adult human lumbar NP (Liebscher *et al.*, 2011), with individual cells isolated within distinct ECM lacuna and separated from any direct cell-cell contact.

1.2.9 Metabolism

The energy demands of IVD cells are met by anaerobic metabolism. Glucose diffuses into the IVD from neighbouring capillary networks and lactic acid diffuses out (Holm *et al.*, 1981; Holm *et al.*, 1982; Ishihara & Urban, 1999).

1.2.10 The Extracellular Matrix of the Intervertebral Disc

The cellular component of IVD tissue accounts for only 0.05% total volume (Urban *et al.*, 2000), the remainder is made up of water and ECM. The ECM in all three IVD regions is composed almost entirely of collagens and proteoglycans. The major proteoglycan of the IVD is aggrecan (Taylor *et al.*, 1992) which, because of its high anionic glycosaminoglycan side chain content (chondroitin and keratin

sulphates) (Roughley *et al.*, 2006), provides the osmotic properties needed for the tissue to resist compression. Variations in the ratio of composition, and so osmotic potential, confer the structural differences between the tissue regions.

Hydroxyproline is a collagen constituent that makes up 10% weight of collagen-α chains (Nimni, 1983). Hydroxyproline content in hydrolysed tissue digests can be quantified using dimethylaminobenzaldehyde (Burleigh *et al.*, 1974), providing a means to determine tissue content of collagens. Sulphated glycosaminoglycans (GAGs) represent the bulk component of IVD proteoglycan molecules (Taylor *et al.*, 1992). GAG content in tissue digests can be determined using 1,9-dimethylmethylene blue (DMMB) dye-binding assay (Farndale *et al.*, 1986), providing a means to determine proteoglycan content. The GAG to hydroxyproline ratio can be used to distinguish differences in ECM constituents and structural properties across IVD tissue regions.

1.2.10.1 Cartilaginous End Plate Extracellular Matrix

The CEP ECM is comprised of type II collagen interspersed with proteoglycans (Antoniou *et al.*, 1996b). The GAG to hydroxyproline ratio of the young adult human lumbar CEP is 2:1 (Mwale *et al.*, 2004). This finding is similar to the GAG/hydroxyproline ratio measured for other hyaline cartilages, including that of the human nasal septum (Homicz *et al.*, 2003) and human talar ankle, distal femur knee and tibial plateau knee cartilages (Treppo *et al.*, 2000). The GAG to hydroxyproline ratio is always below 5 in human hyaline cartilages (Mwale *et al.*, 2004).

Since hydroxyproline represents only 10% weight of collagen, the total collagen content is likely to be around 83% in the young adult human lumbar CEP, with proteoglycans accounting for the remaining 17%. The proteoglycan content is responsible for the weight bearing capacity of the CEP. The osmotic potential it confers is what renders the structure resistant to compressive loads.

1.2.10.2 Annulus Fibrosus Extracellular Matrix

The outer AF ECM is comprised of highly organised type I collagen fibre bundles (Eyre & Muir, 1976) and the inner AF a combination of organised type I and II collagen fibres interspersed with proteoglycans (Eyre & Muir, 1976; Stevens *et al.*, 1979). The GAG to hydroxyproline ratio in the young adult human lumbar AF is 1.6:1 (Mwale *et al.*, 2004). This structural difference with the CEP indicates AF tissue is less capable of resistance to compressive loading, however the concentric ECM organisation renders the tissue resistant to torsion and tension forces (Walmsley, 1953; Adams, 2002).

1.2.10.3 Nucleus Pulposus Extracellular Matrix

The NP ECM consists of an irregular network of type II collagen fibres interspersed with proteoglycans (Stevens *et al.*, 1979). The GAG to hydroxyproline ratio in young adult human lumbar NP is 27:1 (Mwale *et al.*, 2004). Total collagen content is likely to be around 27% with the remaining 73% made up of hydrophilic proteoglycans. The high proteoglycan to collagen ratio explains the gelatinous consistency of the NP compared to the rigid structures observed for CEPs and other hyaline cartilages. This gelatinous consistency allows the temporary deformation required to permit flexion throughout the spine. And further, it confers the ability to generate hydrostatic pressure sufficient to maintain separation of vertebra, even under compressive loading (Figure 1-4).

High proteoglycan content (greater than 50%) is the structural difference that marks NP as a unique tissue type, distinct from hyaline cartilages found elsewhere in the body.

1.2.11 Biomechanics

Compressive loading of the IVD generates hydrostatic pressure in the NP and subsequently, tensile stresses in the AF (Adams, 2002) (Figure 1-5). The balance between swelling pressure in the NP and tension in the AF maintains IVD height and separation of adjacent vertebra (Nachemson & Elfstrom, 1970; Boos *et al.*, 1993).

1.2.12 Tissue Remodelling

ECM in all three tissue regions is synthesised and maintained by local cells, where a continuous process of re-modelling maintains functional integrity of the structures. Collagen and proteoglycan ECM components are synthesised continually (Antoniou *et al.*, 1996a; Antoniou *et al.*, 1996b), this synthesis of new ECM components is balanced by the degradation of old. IVD cells are seen to produce matrix degrading enzymes that facilitate this process and two families have been identified within the IVD; the matrix metalloproteinases (MMPs) and the 'a disintegrin and metalloproteinase with thrombospondin motif's' (ADAMTS's) (Table 1-1).

Native IVD cells mediate ECM remodelling as an adaptive response to changes in the tissue microenvironment. As such, biochemical and mechanical stimuli modulate the process. Biochemical factors include diffusible gaseous signals such as oxygen (Holm *et al.*, 1981; Ishihara & Urban, 1999; Horner & Urban, 2001; Bibby *et al.*, 2005) and the substrates and products of anaerobic metabolism (Holm *et al.*, 1981; Urban *et al.*, 2001; Razaq *et al.*, 2003; Bibby & Urban, 2004; Bibby *et al.*, 2005), mechanical stimuli include the hydrostatic pressures and tensile stresses

Figure 1-4 Schematic Representation of the Structure of Nucleus Pulposus Extracellular Matrix

The nucleus pulposus extracellular matrix is comprised of aggregating proteoglycans enmeshed in an irregular network of collagen fibres. Hyaluronan (dashed line) forms the backbone of the proteoglycan aggregate. This is substituted with aggrecan molecules comprised of a central core protein (open line) and sulphated glycosaminoglycan side chains (solid line). The osmotic potential of glycosaminoglycan side chains causes tissue swelling. In equilibrium this swelling is balanced by tension in the collagen fibre network. Application of a compressive force (C) through the vertebral column forces some of the water out of the NP, effectively increasing aggrecan concentration and swelling potential, and resisting further compression. On removal of the compressive load water is drawn back into the NP and equilibrium restored. Figure adapted from Adams & Roughley (2006).

Figure 1-5 Schematic Representation of the Biomechanics of the Intervertebral Disc
Application of a compressive force (C) through the vertebral column generates hydrostatic pressure (P) in the nucleus pulposus region of the IVD and tensile stresses (T) in the annulus fibrosus. Figure adapted from Adams & Roughley (2006).

Table 1-1 Extracellular Matrix Degrading Enzymes of the Intervertebral Disc

Matrix metalloproteinases (MMPs) exhibit diverse substrate specificity and can be classified broadly into four groups; the *collagenases* that cleave intact interstitial collagen molecules, the *gelatinases* that degrade denatured collagen molecules and basement membranes, the *stromelysins/matrilysins* that digest denatured collagen molecules and non-collagenous matrix proteins, and the *membrane-type MMPs* that modulate enzymatic activity of other MMPs. Some of the 'a disintegrin and metalloproteinase with thrombospondin motif' (ADAMTS) extracellular matrix degrading enzymes have demonstrated aggrecanolytic ability (ADAMTS-1, -4, -5, -8, -9, -15) and distinct family members are seen to produce specific aggrecan cleavage fragments.

Matrix Metalloproteinases		Activity	References
	MMP-1	Collagenase	(Roberts *et al.*, 2000; Doita *et al.*, 2001; Weiler *et al.*, 2002; Le Maitre *et al.*, 2004; Bachmeier *et al.*, 2009; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011)
	MMP-2	Gelatinase	(Crean *et al.*, 1997; Roberts *et al.*, 2000; Weiler *et al.*, 2002; Shen *et al.*, 2003; Rutges *et al.*, 2008; Seguin *et al.*, 2008; Bachmeier *et al.*, 2009; Cui *et al.*, 2010)
	MMP-3	Stromelysin	(Nemoto *et al.*, 1997; Roberts *et al.*, 2000; Doita *et al.*, 2001; Weiler *et al.*, 2002; Shen *et al.*, 2003; Le Maitre *et al.*, 2004; Bachmeier *et al.*, 2009; Millward-Sadler *et al.*, 2009; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011)
	MMP-7	Matrilysin	(Haro *et al.*, 2000; Roberts *et al.*, 2000; Bachmeier *et al.*, 2009; Cui *et al.*, 2010)
	MMP-8	Collagenase	(Roberts *et al.*, 2000; Bachmeier *et al.*, 2009; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011)

A Disintegrin and Metalloproteinase with Thrombospondin Motifs		References
	ADAMTS-1	(Pockert *et al.*, 2009; Cui *et al.*, 2010)
	ADAMTS-2	(Cui *et al.*, 2010)
	ADAMTS-4	(Le Maitre *et al.*, 2004; Hatano *et al.*, 2006; Pockert *et al.*, 2009; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011; Wang *et al.*, 2011)
	ADAMTS-5	(Pockert *et al.*, 2009; Cui *et al.*, 2010; Wang *et al.*, 2011; Zhao *et al.*, 2011)
	ADAMTS-6	(Cui *et al.*, 2010)

	Activity	References		References
MMP-9	Gelatinase	(Crean *et al.*, 1997; Roberts *et al.*, 2000; Weiler *et al.*, 2002; Bachmeier *et al.*, 2009; Millward-Sadler *et al.*, 2009; Tsarouhas *et al.*, 2011)	**ADAMTS-8**	(Pockert *et al.*, 2009)
MMP-10	Stromelysin	(Richardson *et al.*, 2009; Cui *et al.*, 2010)	**ADAMTS-9**	(Pockert *et al.*, 2009)
MMP-11	Stromelysin	(Cui *et al.*, 2010)	**ADAMTS-14**	(Cui *et al.*, 2010)
MMP-13	Collagenase	(Roberts *et al.*, 2000; Le Maitre *et al.*, 2004; Bachmeier *et al.*, 2009; Millward-Sadler *et al.*, 2009; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011)	**ADAMTS-15**	(Pockert *et al.*, 2009)
MMP-14	Membrane-type MMP	(Rutges *et al.*, 2008; Seguin *et al.*, 2008; Cui *et al.*, 2010; Tsarouhas *et al.*, 2011)	**ADAMTS-17**	(Cui *et al.*, 2010)
MMP-16	Membrane-type MMP	(Cui *et al.*, 2010)	**ADAMTS-18**	(Cui *et al.*, 2010)
MMP-19	Stromelysin	(Gruber *et al.*, 2005; Cui *et al.*, 2010)	**ADAMTS-19**	(Cui *et al.*, 2010)
MMP-20		(Cui *et al.*, 2010)		
MMP-21		(Cui *et al.*, 2010)		
MMP-23	Membrane-type MMP	(Cui *et al.*, 2010)		
MMP-24	Membrane-type MMP	(Cui *et al.*, 2010)		
MMP-26	Matrilysin	(Gruber *et al.*, 2012)		
MMP-28		(Cui *et al.*, 2010)		

Table 1-1 Continued from previous page.

experienced during loading and flexion (Ishihara *et al.*, 1996; Handa *et al.*, 1997; MacLean *et al.*, 2005; Wuertz *et al.*, 2009; Barbir *et al.*, 2011; Neidlinger-Wilke *et al.*, 2012).

1.2.13 Aging

The rate of ECM turnover declines in the aging IVD (Antoniou *et al.*, 1996a; Antoniou *et al.*, 1996b; Gruber *et al.*, 2005; Bachmeier *et al.*, 2007; Gruber *et al.*, 2007). This compromises structural integrity (Gruber & Hanley, 2002) and is associated with reduced flexibility and loading capacity and increased susceptibility to injury during normal activity (Prescher, 1998).

1.3 Intervertebral Disc Injury & Disease

1.3.1 Injury

The IVD is susceptible to traumatic and repetitive strain injuries (Figure 1-6). Adult IVDs vary in compressive strength related to individual fitness and lifestyle choices; factors such as obesity and smoking are associated with reduced capacity, whilst regular physical activity is associated with enhanced capacity (Adams *et al.*, 1986). In healthy adults the IVD may resist force applications of up to 13,000 N (Adams & Hutton, 1982).

Outer IVD injury is seen to induce local inflammation, with increased tissue expression of cytokines, up-regulated activity of MMPs (Antoniou *et al.*, 1996a; Kang *et al.*, 1996) and the promotion of scar tissue formation (Nerlich *et al.*, 1997). Post-injury, increased rates of ECM turnover occur (Antoniou *et al.*, 1996a; Duance *et al.*, 1998), although sites of gross injury are never completely remodelled. The original matrix structure is replaced by granulation tissue bridged only in the outermost few millimetres with scar tissue (Melrose *et al.*, 1992; Kaigle *et al.*, 1997). For this reason the IVD is considered to have a limited repair capacity. This may result from an inability to replicate the complex native fibre arrangement laid down during embryonic development, or from low cell density and limited vascular supply that hamper the ability to replace damaged structures.

1.3.2 Prolapse

IVD prolapse is a hyper flexion injury that results from exceeding the physiological limit of flexion or compressive force through a motion segment (Adams & Hutton, 1982; Adams *et al.*, 2000). The IVD is considered to be prolapsed when NP is displaced to an extent that the periphery of the AF is affected. Depending on the extent of displacement, the prolapse can be considered either; an annular bulge or protrusion, where the AF remains intact constraining the NP but has undergone elastic deformation that is not reversed under normal physiological conditions, or extruded, where the CEP or AF has ruptured and NP material is no longer

Figure 1-6 Schematic Representation of Common Types of Intervertebral Disc Injury

Delaminating injuries (A) occur when excessive compressive force applications are made to the annulus fibrosus (AF) and result in shearing of the inter-lamellar bonds. Radial fissures (B) result from repetitive compression and flexion force applications through the motion segment. Most frequently they radiate out from the nucleus pulposus (NP) in a posterior or posterior-lateral direction and displace NP tissue into the AF space. Rim lesions (C) are observed most frequently in the anterior AF and are usually associated with trauma. Figure adapted from Adams & Roughley (2006).

constrained within the IVD space. Sequestration refers to NP material that has been extruded from the IVD and has become detached from it.

1.3.2.1 Spontaneous Resorption

Prolapsed NP may be cleared by spontaneous resorption (Key, 1945). This is more common in lumbar IVD prolapse than in other anatomical regions (Key, 1945; Teplick & Haskin, 1985; Keskil *et al.*, 2004), and even large sequestered NP fragments are seen to disappear in as little as 4 months post displacement (Orief *et al.*, 2012). Exposure of NP tissue to the systemic circulation and inflammatory responses are thought to mediate this process.

1.3.2.2 Inflammatory Response

Inflammation is mediated by the innate immune system in response to tissue infection or injury. Following injury the inflammatory response functions to remove damaged tissue components so that healing can occur. Inflammation is characterised by increased permeability in local vasculature and the influx of fluid, proteins and leukocytes from the circulation to the site of tissue injury (Watkins *et al.*, 1995; Trinchieri, 1999; Letts *et al.*, 2000; Weiss, 2008).

Immune cell infiltrates are found in prolapsed IVD tissue. Studies have characterised these infiltrates in extruded NP as a combination of macrophages (Kanerva *et al.*, 1997; Habtemariam *et al.*, 1998; Kawaguchi *et al.*, 2002; Kobayashi *et al.*, 2009) monocytes (Kawaguchi *et al.*, 2002), T lymphocytes (Kanerva *et al.*, 1997; Habtemariam *et al.*, 1998), B-lymphocytes (Habtemariam *et al.*, 1998) and fibroblasts (Kobayashi *et al.*, 2009). In the case of extruded and sequestered NP, ultrastructural analysis reveals neovascularisation of prolapsed tissue by capillary invasion from the posterior longitudinal ligament (Kobayashi *et al.*, 2009). Neovascularisation enhances immune cell infiltration and migrating monocytes and activated macrophages are often localised to the immediate vicinity of capillary invasion (Kobayashi *et al.*, 2009).

The exact mechanism by which inflammation promotes spontaneous resorption is unclear however, tissue destruction by macrophage mediated enzymatic digestion is implicated. Phagosome presence within the infiltrating macrophages (Kobayashi *et al.*, 2009) indicates phagocytic activity and intracellular digestion, and lysosomal degranulation (exocytosis) indicates secretion of degrading enzymes directly into the ECM to break down intercellular macromolecules (Kobayashi *et al.*, 2009).

1.3.2.3 Autoimmunity

The adaptive immune system may also play a role in spontaneous resorption. It has been shown that exposure of autologus NP to the systemic circulation can activate T and B lymphocytes.

Following embryological development, NP makes no further contact with the blood circulation. This is thought to limit establishment of immunological tolerance to NP derived antigens. It is postulated that subsequent NP exposure to the systemic circulation would induce an autoimmune response (Naylor, 1962). Evidence in support of this theory was first presented by Bobechko & Hirsch, in 1965 who observed that exposure of autologus NP to the systemic circulation stimulates the production of auto-immune antibodies in the primary lymph nodes of laboratory animals. Since then immunoreactivity to prolapsed NP has been indicated by the presence of antigen-antibody complexes (Marshall *et al.*, 1977; Habtemariam *et al.*, 1998; Satoh *et al.*, 1999). And further animal studies have shown that autologus NP attracts activated lymphocytes (T and B cells) (Geiss *et al.*, 2007) and stimulates differentiation of the T-helper subset towards a T_H2 effector phenotype (Geiss *et al.*, 2009).

This adaptive immune system response to exposed NP tissue is little studied and the contribution it may make to spontaneous resorption is unclear. Presumably, T cell mediated tissue destruction would enhance spontaneous resorption and further promote the inflammatory response of the innate immune system.

Other proposed mechanisms by which spontaneous resorption may occur are tissue shrinkage by dehydration (Hirabayashi *et al.*, 1990; Slavin *et al.*, 2001) and protrusion regression by retraction of the AF (Guinto *et al.*, 1984; Teplick & Haskin, 1985). Native cells within prolapsed NP tissue may also mediate the breakdown of displaced tissue, increased expression of matrix degrading enzymes is observed (Kang *et al.*, 1996; Kang *et al.*, 1997) alongside increased rates of cellular apoptosis (Wang *et al.*, 2011).

1.3.3 Intervertebral Disc Degeneration

IVD degeneration is a non-inflammatory arthropathy that affects the fibro-cartilaginous joints of the spine. A characteristic pattern of morphological and histological changes occur that lead to disorganisation of ECM and structural failure. It is thought that these changes are mediated by disturbances in the biology of IVD cells (Freemont *et al.*, 2002).

1.3.3.1 Cell Phenotype and Viability

Decreased cell viability is observed within degenerate IVDs (Horner & Urban, 2001; Bibby *et al.*, 2002; Johnson & Roberts, 2003; Bibby & Urban, 2004; Gruber *et al.*, 2005; Roberts *et al.*, 2006; Le Maitre *et al.*, 2007c). This has been assessed by expression of the transcription factor, SOX9, that is down-regulated in degeneration (Gruber *et al.*, 2005). SOX9 modulates chondrogenesis in development (Healy *et al.*, 1999; Huang *et al.*, 2001; Lefebvre *et al.*, 2001; Kronenberg, 2003) and modulates type II collagen and aggrecan expression (Lefebvre *et al.*, 1997; Huang *et al.*, 2002; Han & Lefebvre, 2008), which is both essential for IVD health and characterises the native cell population (Chelberg *et al.*, 1995). Down-regulation of SOX9 indicates that cells present within degenerate IVDs may no longer be of a native cell phenotype.

An increased percentage of senescent and apoptotic cells are also observed within the degenerate cell population (Park *et al.*, 2001a; Roberts *et al.*, 2006; Gruber *et al.*, 2007; Le Maitre *et al.*, 2007c; Tschoeke *et al.*, 2008; Wang *et al.*, 2011). Whilst senescent cells may persist within tissues, they are often observed to exhibit a catabolic phenotype (Coppe *et al.*, 2008) and increased rates of cellular apoptosis would indicate a declining cell population. However, tissue cellularity is maintained in the degenerate IVD (Liebscher *et al.*, 2011) by replication of surviving non-senescent cells within lacuna that give rise to cell clusters (Johnson & Roberts, 2003).

1.3.3.2 Anabolic Metabolism

Anabolic metabolism of the cells present within the degenerate IVD is altered from that in the normal. Synthesis of aggrecan and type II collagen is decreased and synthesis of type I collagen is increased (Antoniou *et al.*, 1996a). This alters the ECM structure in all 3 IVD tissue regions (Antoniou *et al.*, 1996a; Antoniou *et al.*, 1996b), hydration is reduced and gelatinous structures become increasingly fibrous. The stimulus for this altered metabolism is not known however, presumably senescent cells and cells that exhibit an altered phenotype no longer contribute to correct ECM synthesis.

1.3.3.3 Catabolic Metabolism

Catabolic metabolism of the cells present within the degenerate IVD is altered from that seen in the normal. Expression of matrix degrading enzymes is increased (Weiler *et al.*, 2002; Le Maitre *et al.*, 2004; Gruber *et al.*, 2005; Le Maitre *et al.*, 2007a; Hoyland *et al.*, 2008; Rutges *et al.*, 2008; Bachmeier *et al.*, 2009; Richardson *et al.*, 2009; Zhao *et al.*, 2011) and localised to areas of altered tissue morphology (Weiler *et al.*, 2002). Matrix degrading enzyme activity correlates

positively with increasing severity of degenerative tissue changes (Weiler *et al.*, 2002; Le Maitre *et al.*, 2004; Le Maitre *et al.*, 2007a; Pockert *et al.*, 2009).

1.3.3.4 Aetiology

The order in which cellular changes occur in IVD degeneration is poorly understood. There is considerable evidence to link decreased cell viability and dysregulated metabolism with impaired diffusion through the IVD, although whether this is a cause of alterations in native cell biology or a consequence of it, is yet to be established. Decreased permeability of the bony and cartilaginous endplates is observed alongside IVD degeneration (Nachemson *et al.*, 1970) and limits the diffusion of nutrients to cells within the IVD and the diffusion of metabolic waste away from them. Nutrient supply is critical to IVD cell survival and whilst transient episodes of decreased glucose may be tolerated, low concentrations maintained for several days result in cell death (Horner & Urban, 2001; Bibby & Urban, 2004; Bibby *et al.*, 2005). The accumulation of waste from anaerobic metabolism (lactic acid) results in an increasingly acidic cellular micro-environment (Diamant *et al.*, 1968; Kitano *et al.*, 1993) and low pH (<6.4) has a further adverse effect on cell viability (Horner & Urban, 2001; Bibby *et al.*, 2005) and reduces rates of ECM synthesis (Razaq *et al.*, 2003). Reduced oxygen tension is also seen to impair rates of ECM synthesis (Ishihara & Urban, 1999; Horner & Urban, 2001). However, whilst impaired diffusion may reduce cell viability and rates of ECM synthesis, the altered anabolic activities of native cells, especially the increased production of type I collagen results in an increasingly fibrous ECM. This in itself impairs rates of diffusion, particularly through the CEP and NP. It's therefore unclear whether impaired diffusion contributes to altered cell biology or altered cell biology results in impaired diffusion.

Similarly, the effects of low oxygen tension are not understood, since little energy is derived from oxidative phosphorylation and the IVD cells energy requirements are met by glycolysis even in the presence of oxygen (Ishihara & Urban, 1999; Horner & Urban, 2001). However, the loss of effective transport routes across the CEPs does correlate with decreased cell viability within the IVD (Urban *et al.*, 2001; Bibby *et al.*, 2002).

Increased rates of catabolic metabolism are attributed in part to the increased percentage of senescent cells that produce MMPs and ADAMTSs (Le Maitre *et al.*, 2007c). However, remaining non-senescent cells also contribute to increased catabolism within the IVD, and whilst nutrient deprivation reduces anabolic metabolism in these cells, it has no effect on the rate at which active matrix

degrading enzymes are produced (Razaq *et al.*, 2003). This may represent a mechanism by which catabolism overtakes anabolism and results in the characteristic ECM depletion.

There is also considerable evidence to suggest a genetic predisposition to IVD degeneration. Genetic polymorphisms associated with structural components of the IVD, ECM degrading enzymes and biochemical regulators of IVD cell biology such as cytokines and growth factors, are implicated in the onset and progression of IVD degeneration (Table 1-2).

1.3.3.5 Structural Failure of the Degenerate Intervertebral Disc

Over time, the imbalance between anabolic and catabolic activity compromises the structural integrity of the IVD (Figure 1-7). Decreased aggrecan concentration reduces osmotic potential and dehydration occurs (Antoniou *et al.*, 1996a; Antoniou *et al.*, 1996b). This is first evident in the NP where the ability to generate hydrostatic pressure is compromised (Wenger *et al.*, 2005). Progressively, the NP condenses to form a more fibrous structure and IVD height is reduced. This process is exacerbated by the incorporation of type I collagen fibres (Antoniou *et al.*, 1996a; Nerlich *et al.*, 1997) and demarcation between NP and AF is lost (Nerlich *et al.*, 1997; Prescher, 1998; Gruber & Hanley, 2002). Dehydration and type I collagen fibre incorporation is also evident in the CEP (Antoniou *et al.*, 1996b). These changes reduce diffusion rates (Nachemson *et al.*, 1970), particularly of water soluble nutrients.

1.3.3.6 Biomechanics of the Degenerate Intervertebral Disc

Reduced IVD height alters the biomechanics of the motion segment (Adams, 1996; Adams, 2002). Of the IVD tissues, the AF is least resistant to compressive loading. Dehydration of the inner AF (Antoniou *et al.*, 1996a; Adams, 2002) further reduces this capacity. Hydrostatic pressure in the NP is required to generate tension in the concentric lamella of the AF and to maintain their correct alignment (Peacock, 1952; Walmsley, 1953). In the absence of this hydrostatic pressure the lamella 'sag', bulging outward, and the AF is subjected to compressive forces during normal movement (Adams *et al.*, 1996a). The degenerate IVD functions increasingly as a fibrous 'pad' to withstand loading forces and the biomechanical properties are greatly reduced compared to those of the normal IVD (Adams *et al.*, 1996a; Ebara *et al.*, 1996).

Permeative slits, or fissures appear in the condensed NP (Nerlich *et al.*, 1997; Prescher, 1998). Fissure formation correlates with increased rates of catabolism (Le Maitre *et al.*, 2004; Le Maitre *et al.*, 2007a; Pockert *et al.*, 2009) and cells

Polymorphism in Gene Encoding:	References
Structural Components of the IVD	
Collagen fibers (II & IX)	(Solovieva *et al.*, 2002; Pluijm *et al.*, 2004; Tilkeridis *et al.*, 2005; Solovieva *et al.*, 2006; Mio *et al.*, 2007; Videman *et al.*, 2009; Nakki *et al.*, 2011)
Aggrecan	(Kawaguchi *et al.*, 1999; Solovieva *et al.*, 2007; Videman *et al.*, 2009)
HAPLN1	(Urano *et al.*, 2011)
Matrix Degrading Enzymes	
MMP-2	(Dong *et al.*, 2007)
MMP-3	(Takahashi *et al.*, 2001)
MMP-9	(Hirose *et al.*, 2008; Sun *et al.*, 2009)
Biochemical Regulators of IVD Cell Biology	
Interleukin-1	(Solovieva *et al.*, 2004; Solovieva *et al.*, 2006)
Interleukin-6	(Noponen-Hietala *et al.*, 2005)
IGF-1 receptor	(Urano *et al.*, 2008)
Vitamin D receptor	(Videman *et al.*, 1998; Kawaguchi *et al.*, 2002a; Cheung *et al.*, 2006; Chen *et al.*, 2012)

Table 1-2 Genetic Polymorphisms Associated with Intervertebral Disc Pathology

MMP; matrix metalloproteinase; IGF; insulin-like growth factor; HAPLN1, hyaluronan and proteoglycan link protein-1.

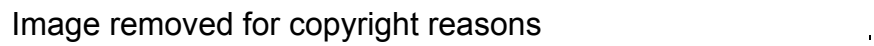


Figure 1-7 Characteristic Macroscopic Structural Changes Associated with Intervertebral Disc Degeneration

Images shown are photographs of cadaveric human lumbar intervertebral discs (IVDs) in the mid-sagittal plane. (A) The young adult lumbar IVD; the central gelatinous nucleus pulposus (NP) is clearly demarcated from the organised lamellar structure of the annulus fibrosus (AF). (B) Mild IVD degeneration; increased incorporation of type I collagen fibres results in a more fibrous NP. (C) Moderate IVD degeneration; in the absence of hydrostatic pressure in the NP the lamella of the inner AF may collapse into the NP space (red arrow). (D) Severe IVD degeneration; demarcation between the NP and AF is lost and IVD height is reduced. (E) Posterior extrusion IVD prolapse; NP has extruded through the ruptured posterior AF and is constrained between the vertebral body and the posterior longitudinal ligament (red arrow). Figure adapted from Adams & Roughley (2006).

producing matrix degrading enzymes are localised to the vicinity of tissue damage (Weiler *et al.*, 2002). Progressively, the fissures radiate outward and into the AF where inner lamella may collapse into the NP space (Adams & Hutton, 1985; Osti *et al.*, 1992; Osti *et al.*, 1992). The mechanisms of fissure formation are poorly understood, whether the compressive loading of dehydrated, weaker IVD tissue results in fissure formation and the increased cellular catabolic activity is an attempt to remodel damaged ECM, or whether regions of excessive cellular catabolic activity destroy ECM sufficiently for fissures to appear remains unclear.

1.3.3.7 Vasculature and Innervation

In severely degenerate IVDs, proliferation of blood vessels and accompanying sensory nerve fibres occurs in the CEP region (Brown *et al.*, 1997). Vascularisation in the AF is also increased with the appearance of vertically oriented arteries (Kauppila, 1995). This neovascularisation has been traced to the CEP through which new vessels penetrate the IVD from adjacent vertebral bodies (Freemont *et al.*, 2002). Sensory nerve fibre in-growth is seen to follow the same route and vascular endothelial expression of nerve growth factors is associated with this process (Freemont *et al.*, 1997; Freemont *et al.*, 2002).

1.3.4 The Relationship between Injury and Degeneration

The relationship between IVD injury and degeneration is not well understood. Each can be observed independently however, frequently evidence of both is apparent in pathological tissues. Injury occurs to the IVD through trauma that exceeds the physiological limit of the tissues strength (Adams & Hutton, 1982). Annular tear and delaminating injuries are seen in IVDs from young individuals that show no signs of degeneration (Hirsch & Schajowicz, 1953; Jensen *et al.*, 1994). However, degenerative changes weaken the IVD and are associated with increased susceptibility to certain injuries, particularly radial fissures (Adams & Hutton, 1985; McNally *et al.*, 1993). Prolapse is linked to mild and moderate levels of degeneration, through radial fissures that provide the route down which NP is displaced (Adams & Hutton, 1985).

Similarly, degeneration occurs in IVDs that show no signs of previous injury (Jensen *et al.*, 1994). Although certain injuries are associated with the instigation of degenerative changes, particularly injuries to the end plates (Adams *et al.*, 2000). Typically, these result in NP decompression and are followed by rapid degenerative changes (Kaigle *et al.*, 1997). IVD degeneration increases susceptibility to delaminating injuries that result from compressive loading of the AF (Adams *et al.*, 1996b; Ebara *et al.*, 1996), however, prolapse is inhibited once hydration of the NP is lost (Adams & Hutton, 1982).

1.3.5 Loading and Intervertebral Disc Degeneration

Historical loading patterns appear to be an environmental risk factor associated with IVD degeneration (Adams *et al.*, 2000). Obesity and manual occupations increase loading through the IVDs and are associated with an enhanced risk of IVD degeneration development (Solovieva *et al.*, 2002). Since native IVD cells mediate ECM remodelling as an adaptive response to mechanical stimuli (Ishihara *et al.*, 1996; Handa *et al.*, 1997; MacLean *et al.*, 2005; Wuertz *et al.*, 2009; Barbir *et al.*, 2011; Neidlinger-Wilke *et al.*, 2012), which is considered beneficial to IVD structural integrity, efforts to understand the detrimental effects of excessive loading have examined the biologic responses of the IVD to force applications (Wang *et al.*, 2007; Walter *et al.*, 2011). Key findings from these investigations are that excessive or complex loading patterns detrimentally modulate expression of biochemical regulators of native IVD cell biology, such as growth factors and cytokines. Growth factors, particularly transforming growth factor-β (TGF-β), are suggested to promote anabolic metabolism by native IVD cells (Nishida *et al.*, 1999) while cytokines such as Interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α), are suggested to promote catabolic metabolism (Bachmeier *et al.*, 2007; Le Maitre *et al.*, 2007a; Le Maitre *et al.*, 2007d; Hoyland *et al.*, 2008). It is postulated that IVD degeneration is induced by abnormal loading and mediated by cytokines (Freemont *et al.*, 2001).

1.4 Cytokines

Cytokines are a large and diverse group of peptide molecules that act as messages in the bodies' intercellular communications network. They convey signals between local cells to modulate physiological responses to altered or adverse micro-environmental conditions (Hancock, 2005). They are produced by many cell types in response to a wide variety of stimuli.

Cellular perception of stimuli that requires a cytokine mediated response results in the secretion or extracellular membrane presentation of a particular cytokine. This signal can then be perceived by another 'target' cell that possesses the specific receptor molecule (Dinarello & Moldawer, 2000). Signal transduction, which is transfer of the signal through several intracellular components, is then often required to convey the message to its terminal destination within the cell (Hancock, 2005). A response is elicited so that the outcome is appropriate to the original stimuli or stress.

Cytokine extracellular signals are generally confined to the locality in which they are produced. Extracellular membrane presentation limits their range to adjacent cells and secretion (or in some cases 'shedding' of membrane bound cytokines) permits

only short range signal diffusion. This is because cytokines released into the extracellular medium are rapidly degraded by extracellular proteases, sequestered within the ECM or taken up by neighbouring cells (Hancock, 2005). As such, cytokines generally modulate only local cellular responses.

Autocrine, paracrine and cell-cell contact form the basis of cytokine intercellular signalling mechanisms (Hancock, 2005). Diffusible signals produced and perceived by the same cell are autocrine, and a means of the cell signalling to itself. Paracrine and cell-cell contact facilitate communication between different cells; diffusible signals released by one cell and perceived by another are paracrine, and receptor mediated perception of membrane bound cytokines or diffusion through plasma membrane gap junctions are cell-cell contact mechanisms.

Cytokines are produced within the IVD by native cells. Among those identified some, particularly those associated with the pathogenesis of IVD degeneration, have been characterised. These include IL-1 and TNF-α.

1.4.1 Interleukin-1

Seven members make up the IL-1 family. Two related but distinct genes encode the agonists, IL-1α and IL-1β. Both cytokines are synthesised as 31kDa precursor molecules that remain in the cytosol after translation (Andersson *et al.*, 1992). Post-translational cleavage results in the mature 17kDa peptides (Black *et al.*, 1988; Stevenson *et al.*, 1992).

The IL-1α precursor molecule, proIL-1α, has full biological activity (Mosley *et al.*, 1987) and can be targeted for secretion or membrane presentation (Kurt-Jones *et al.*, 1985). ProIL-1α is constitutively expressed by cells in several organs including skin, liver, lungs and kidneys and accumulates in the cytosol (Andersson *et al.*, 1992). In healthy tissues, proIL-1α functions to promote homeostasis and in skin this is an autocrine effect (Hauser *et al.*, 1986; Maier *et al.*, 1990). In disease or inflammation, cell necrosis releases cytosolic proIL-1α to act as a paracrine signal on neighbouring cells (Chen *et al.*, 2007; Cohen *et al.*, 2010).

In contrast, the IL-1β precursor molecule, proIL-1β, is not biologically active (Mosley *et al.*, 1987). Intracellular post-translational modification by another IL-1 family member, IL-1 converting enzyme (ICE), is required to generate the mature peptide (Black *et al.*, 1988). ProIL-1β accumulates in the cytosol (Andersson *et al.*, 1992), cleavage and activation occurs at the inner surface of the cell membrane and triggers secretion from the cell (Dinarello & Moldawer, 2000). IL-1β is not presented on the extracellular plasma membrane and is not constitutively

expressed in healthy tissues. Both IL-1 agonists are powerful inducers of the innate immune systems inflammatory response, and regulators of other cytokines involved in this process (Dinarello & Moldawer, 2000).

Perception of IL-1 signalling is by the functional IL-1 family receptor, IL-1RI, that forms a heterodimeric complex with the IL-1 receptor accessory protein, IL-1RAcP, to facilitate signal transduction (Greenfeder *et al.*, 1995). A second IL-1 receptor exists, IL-1RII, that sequesters ligands without transducing a signal, and is often referred to as a 'decoy' receptor (Colotta *et al.*, 1993; Sims *et al.*, 1993) (Figure 1-8).

The final member of the IL-1 family is IL-1 receptor antagonist, IL-1Ra. IL-1Ra is a 22kDa glycosylated peptide molecule (Seckinger *et al.*, 1989; Hannum *et al.*, 1990; Mazzei *et al.*, 1990) that is secreted into the extracellular medium (Arend *et al.*, 1998). IL-1Ra binds receptor IL-1RI without triggering signal transduction (Dripps *et al.*, 1991). Receptor occupancy by IL-1Ra effectively blocks signalling by both IL-1α and IL-1β.

1.4.1.1 Interleukin-1 Intracellular Signalling

Almost all biological effects of IL-1 are mediated by inducing the expression of target genes. As such, IL-1 signalling results in the production of transcription factors that drive expression of inducible genes. The IL-1 signalling receptor, IL1-RI, is a trans-membrane protein expressed by a wide variety cells (Saklatvala & Guesdon, 1991; Kracht & Saklatvala, 2002). Agonist induced association of IL-1RI with IL-1RAcP initiates the formation of an intracellular protein complex, whose main component is the adaptor protein, TRAF6, a member of the TNF-Receptor-Associated Factor family (Jiang *et al.*, 2002). The TRAF6 complex contains active forms of IκB kinases (IKKs) and certain members of the mitogen activated protein kinases (MAP3K) family, such as TGF-β-activated kinase (TAK1) and MEKK3 (Jiang *et al.*, 2002). The IKKs phosphorylate IκB, the inhibitor of Nuclear Factor (NF)-κB, triggering degradation of IκB and release of the p50/p65 heterodimeric transcription factor NF-κB. The MAP3Ks activate three subtypes of mitogen activated protein kinases (MAPKs), Erk, JNK and p38, which in turn activate transcription factors such as ELK-1, AP1 or ATF2 (Kracht & Saklatvala, 2002).

IL-1RI may also activate the phosphatidyl-inositol-3 kinase (PI3K) pathway by two mechanisms (Davis *et al.*, 2006; Wang *et al.*, 2006; Liu *et al.*, 2007; Parhar *et al.*, 2007); the first via TRAF complex activation of the tyrosine kinase, c-src (Gelman *et al.*, 2006; Wang *et al.*, 2006), and the second via direct activation of the P13K subunit, p85, by IL-1RI (Davis *et al.*, 2006). PI3K activates 3-phosphoinositide-

dependent protein kinase 1 (PDK1) which may then trigger either or both; IκB degradation and release of NF-κB and/or activation of protein kinase B and subsequent Akt transcription factor activity (Liu *et al.*, 2007; Parhar *et al.*, 2007).

1.4.2 Tumour Necrosis Factor-α

TNF-α is synthesised as a 26kDa protein that is biologically active and displayed on the extracellular plasma membrane (Kriegler *et al.*, 1988). Membrane bound TNF-α may signal to adjacent cells displaying receptors through cell-cell contact but also functions as a paracrine signal when 'shed' from the plasma membrane. TNF-α converting enzyme (TACE/ADAM-17) is a membrane bound 'a disintegrin and metalloproteinase' (ADAM) that cleaves the extracellular domain of membrane bound TNF-α to release a biologically active 17kDa soluble TNF-α molecule (Black *et al.*, 1997; Moss *et al.*, 1997).

TNF-α is produced by many cell types and in response to a broad range of stimuli. Biologically, it modulates cellular proliferation, differentiation, metabolism, and can induce apoptosis (Zhang & Tracey, 1998). TNF-α is also a powerful inducer of the innate immune systems inflammatory response, and a regulator of other cytokines involved in this process (Dinarello & Moldawer, 2000).

Responses to TNF-α are mediated by ligand binding to two types of transmembrane glycoprotein receptors, TNF receptor I (TNF-RI/TNF-R55) and TNF receptor II (TNF-RII/TNF-R75) (Figure 1-9). Both receptors are constitutively expressed in all cells but are susceptible to cleavage by MMPs. Inflammatory signals can induce this cleavage and 'shedding' of the receptors' extracellular domain (Higuchi & Aggarwal, 1994; Solorzano *et al.*, 1997), shed receptors retain binding affinity and may then function as inhibitors of TNF-α activity.

1.4.3 Interleukin-1 and Tumour Necrosis Factor-α in the Normal Intervertebral Disc

Stimuli that result in cytokine expression within the normal IVD are unknown, as are the exact roles that cytokines play in IVD cell biology. However, cytokine expression occurs within the normal IVD and so it can be assumed that the stimuli form part of the normal physiological conditions to which native cells are exposed.

IL-1α, IL-1β, IL-1Ra, IL-1RI and ICE are expressed by native cells of the IVD (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d). Expression patterns of the agonists are similar (Le Maitre *et al.*, 2005), and their function may be maintenance of tissue homeostasis through modulation of anabolic and catabolic cell activities. Characteristic *in vitro* responses of human NP cells to agonist stimulation include suppression of aggrecan transcription without affecting type I or II collagen

Figure 1-8 Schematic Representation of Interleukin-1 Family Expression

From left to right: IL-1β (or –1α, not shown) binds to functional cell bound IL-1 receptor, IL-1RI. This binding triggers conformational changes in the receptor and leads to the recruitment of IL-1 receptor accessory protein, IL-1RAcP, and the formation of the signalling receptor heterodimeric complex. The intracellular domains of both subunits contain Toll domains that are important for signal transduction, IL-1 receptor activating kinase (IRAK) is recruited to close proximity of the Toll domains and this leads to signal transduction and the activation of nuclear genes. The IL-1 decoy receptor, IL-1RII possesses a short intracellular domain that lacks a Toll domain and does not signal. IL-1Ra competes with IL-1β binding to IL-1RI. Receptors occupied by IL-1Ra do not form the signalling heterodimeric complex and hence do not signal. Binding of IL-1β to the shed domains of receptors, soluble IL-1RI (sIL-1RI) and soluble IL-1RII (sIL-1RII) sequesters the ligand and prevents binding to membrane bound IL-1RI.

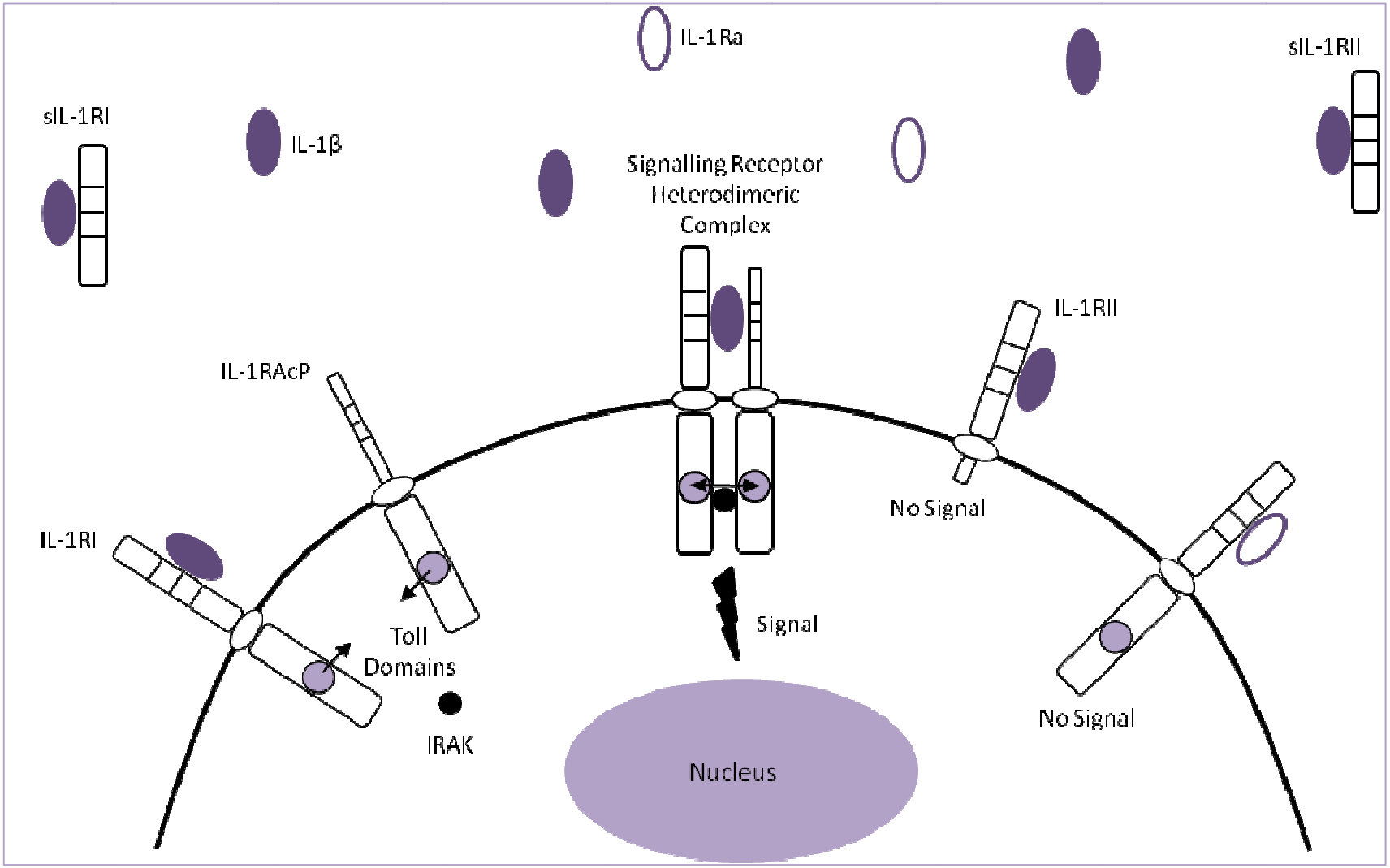
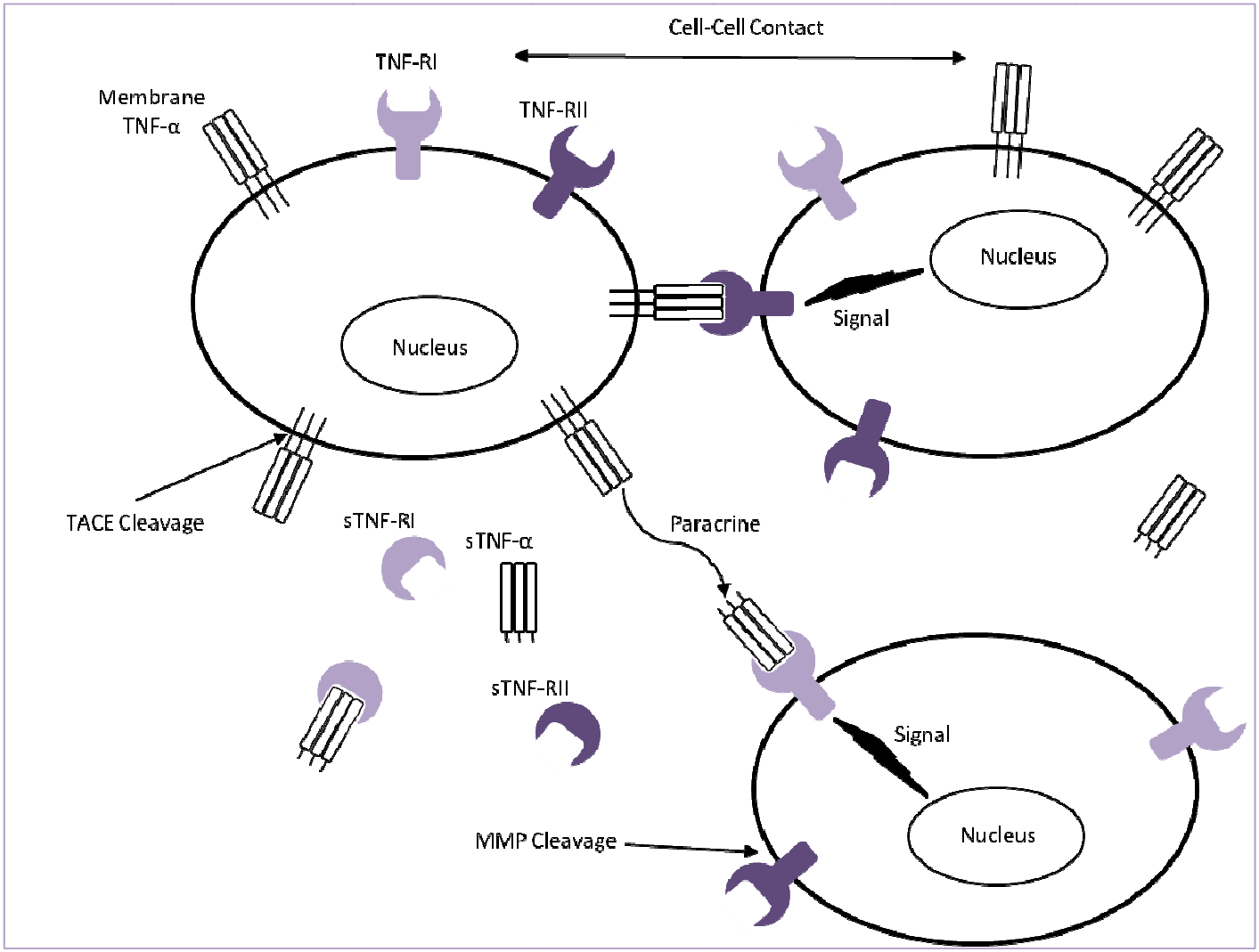

IL-1Ra
sIL-1RII
sIL-1RI
IL-1β
Signalling Receptor
Heterodimeric
Complex
IL-1RII
IL-1RAcP
No Signal
IL-1RI
Signal
Toll
Domains
IRAK
No Signal
Nucleus

Figure 1-9 Schematic Representation of Autocrine, Juxtacrine and Paracrine Signalling Mechanisms of TNF-α

Top left to right: TNF-α is a biologically active homotrimeric protein that is presented on the plasma membrane. TNF receptor I (TNF-RI) and TNF receptor II (TNF-RII) are constitutively expressed in all cells. Membrane bound TNF-α may signal to adjacent cells displaying receptors through cell-cell contact (juxtacrine) mechanisms. Bottom: Membrane bound TNF-α is susceptible to cleavage by TNF-α converting enzyme (TACE) which releases a biologically active 17kDa soluble form of TNF-α (sTNF-α) which may then act as a paracrine signal on neighbouring cells. Under certain conditions the extracellular domain of TNF-RI and TNF-RII can be 'shed' by matrix metalloproteinase cleavage. Shed receptors retain binding affinity and may then function to sequester ligands and prevent binding to signalling receptors.


Cell-Cell Contact
TNF-RI
Membrane
TNF-α
TNF-RII
Nucleus
Nucleus
Signal
TACE Cleavage
sTNF-RI
sTNF-α
Paracrine
sTNF-RII
Signal
Nucleus
MMP Cleavage

(Le Maitre *et al.*, 2005) and increased MMP -3, -9 and -13 transcription (Le Maitre *et al.*, 2005; Millward-Sadler *et al.*, 2009) without affecting that of ADAMTS -4 and -5 (Le Maitre *et al.*, 2005).

TNF-α, TNF-RI, TNF-RII and TACE are expressed by native cells of the IVD (Weiler *et al.*, 2005; Bachmeier *et al.*, 2007; Le Maitre *et al.*, 2007d). Expression is greater in the NP than in the AF and increases with aging (Bachmeier *et al.*, 2007). TNF-α may also function to maintain homeostasis through modulation of anabolic and catabolic cell activities and through modulation of IL-1 expression. Characteristic *in vitro* responses of human NP cells to TNF-α stimulation include suppression of type I and II collagen transcription without affecting that of aggrecan (Millward-Sadler *et al.*, 2009) and increased MMP -3 and -13 transcription without affecting that of MMP-9 (Millward-Sadler *et al.*, 2009).

1.4.3.1 Interleukin-1 and Tumour Necrosis Factor-α Inter-Regulation

The subtle differences in modulatory potential of these two cytokines may function to maintain the correct balance of ECM composition. An inter-regulatory relationship exists between IL-1 and TNF-α, with IL-1 stimulation having no effect on transcription of IL-1 family members (Le Maitre *et al.*, 2005) but increasing the transcription of TNF-α and receptor TNF-R75, without affecting that of receptor TNF-R55 (Millward-Sadler *et al.*, 2009). TNF-α stimulation increases transcription of IL-1β and IL-1Ra without affecting IL-1α or receptor IL-1RI (Millward-Sadler *et al.*, 2009) and increases its own transcription alongside receptor, TNF-R75, without affecting TNF-R55 (Millward-Sadler *et al.*, 2009). The ability of TNF-α to modulate both its own transcription and that of IL-1 suggests that TNF-α has greater regulatory potential within the IVD however, catabolic responses to IL-1 stimulation are greater than those observed for TNF-α indicating that IL-1 has a greater potential to instigate catabolic changes.

1.4.4 Interleukin-1 and Tumour Necrosis Factor-α in the Degenerate Intervertebral Disc

In degenerate IVDs, expression of IL-1α, IL-1β, IL-1RI and ICE is greater than that seen in non-degenerate (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d), whilst expression of the antagonist, IL-1Ra, is unchanged (Le Maitre *et al.*, 2005). Increased agonist expression correlates positively with increasing severity of degeneration (Le Maitre *et al.*, 2005) as does the expression of MMPs (Weiler *et al.*, 2002), however, a direct link to show that increased IL-1 expression causes the increased MMP expression *in vivo* is yet to be established. *In situ* zymography of human IVD tissue shows that matrix degrading enzyme activity is greatly enhanced in both normal and degenerate samples following IL-1 stimulation, and suppressed

to levels much lower than those seen in un-stimulated controls by the antagonist, IL-1Ra (Le Maitre *et al.*, 2007b; Hoyland *et al.*, 2008). Since increased expression of the IL-1 agonists in degenerate NP cells is seen without a concordant increase in IL-1Ra (Le Maitre *et al.*, 2005), an imbalance between agonist and antagonist is thought to form the mechanism by which IL-1 may cause increased matrix degradation.

The characteristic *in vitro* responses of human NP cells derived from degenerate tissue, to IL-1 agonist stimulation, are altered compared to those in NP cells derived from the normal IVD. Modulation of anabolic genes is by suppression of type I collagen (Le Maitre *et al.*, 2005), type II collagen (Studer *et al.*, 2011) and aggrecan (Studer *et al.*, 2011) transcription. Modulation of catabolic genes is by increased MMP -3, -9, -13 (Millward-Sadler *et al.*, 2009) and ADAMTS-4 transcription (Le Maitre *et al.*, 2005) without affecting ADAMTS-5 (Le Maitre *et al.*, 2005). This indicates that the modulatory potential of IL-1 is altered, non-specific suppression of anabolic gene transcription occurs and an ability to modulate ADAMTS expression develops. These changes in cellular response to IL-1 stimulation would enhance the drift towards a catabolic microenvironment and ECM depletion.

Expression of TNF-α, TNF-R75 and TACE is also greater in the degenerate IVD than in the non-degenerate (Bachmeier *et al.*, 2007; Le Maitre *et al.*, 2007d). Conflicting reports of receptor, TNF-R55, protein expression exist however, with some studies indicating expression is increased (Bachmeier *et al.*, 2007) and others reporting no change when comparing degenerate to non-degenerate IVDs by the same immuno-detection technique (Le Maitre *et al.*, 2007d). TNF-R55 transcription has been assessed and found to be consistent in both non-degenerate and degenerate IVDs (Le Maitre *et al.*, 2007d; Tschoeke *et al.*, 2008), and it may be that the differences reported in protein expression relate to antibody specificity and fragment detection since this receptor is known to be susceptible to MMP cleavage (Higuchi & Aggarwal, 1994; Solorzano *et al.*, 1997). Increased TNF-α expression correlates positively with increasing severity of degeneration (Bachmeier *et al.*, 2007) however, the modulatory potential of TNF-α on catabolic metabolism appears to be reduced, indicating that increased TNF-α expression may not account for the increased MMP expression in the degenerate IVD. This is demonstrated by the *in vitro* responses of human NP cells derived from degenerate tissue, to TNF-α stimulation, which are altered to those in cells derived from the normal IVD. Modulation of catabolic genes is by increased MMP-3 transcription

without affecting that of MMP -9 or -13 (Millward-Sadler *et al.*, 2009). The modulatory effect on MMP-13 observed in NP cells from non-degenerate IVDs is lost and whilst up-regulation of MMP-3 transcription still occurs, the response observed (fold increase) is less than that seen in cells derived from the non-degenerate IVD (Millward-Sadler *et al.*, 2009). This is confirmed by *in situ* zymography of human IVD tissue, that shows TNF-α stimulation of non-degenerate samples results in mildly increased matrix degrading enzyme activity that can be reduced to slightly below the level seen in un-stimulated controls by anti-TNF stimulation (Hoyland *et al.*, 2008), but that stimulation of degenerate samples with either TNF-α or anti-TNF has no effect on matrix degrading enzyme activity (Hoyland *et al.*, 2008). This indicates either that TNF-α exerts a much lesser modulatory effect on catabolic metabolism in the degenerate IVD, or that the regulatory mechanism that prevents excessive TNF-α mediated catabolism remains functional within the degenerate IVD.

In vitro, modulation of anabolic genes in response to TNF-α stimulation is also altered in NP cells derived from degenerate IVDs compared to those from non-degenerate counterparts. The response is characterised by suppression of type I collagen (Millward-Sadler *et al.*, 2009), type II collagen (Studer *et al.*, 2011) and aggrecan (Studer *et al.*, 2011) transcription. This indicates that differential modulation of ECM components is replaced by non-specific suppression of anabolic gene transcription. Increased TNF-α expression in the degenerate IVD may exert detrimental modulatory effects on ECM integrity by down-regulating anabolic metabolism rather than adversely modulating catabolic metabolism.

The inter-regulatory effects of the two cytokines are also altered in degeneration. TNF-α up-regulates its own transcription and that of IL-1 in a similar manner to that seen in the normal IVD. Presumably this process is itself modulated since excessive cytokine expression does not occur in the normal IVD and TNF-α up-regulates IL-1 agonist and antagonist to similar extents, maintaining equilibrium. However, the regulatory potential of IL-1 is greatly increased. IL-1 stimulation up-regulates transcription of both IL-1 agonists without affecting that of the antagonist indicating a positive feedback loop is established in NP cells derived from degenerate tissue (Le Maitre *et al.*, 2005).

Together these findings implicate dysregulated cytokine expression in the pathogenesis of IVD degeneration. They indicate that the characteristic cellular changes – reduced anabolic metabolism and increased catabolic metabolism, may result directly from this dysregulation, and that the endogenous inhibitor of IL-1, IL-

1Ra may abolish excessive catabolism in the degenerate IVD. Further evidence to support this theory in the human IVD is shown by the introduction of autologus NP cells, transfected to over express IL-1Ra, into degenerate NP tissue explants, where MMP and ADAMTS-4 expression was reduced (Le Maitre *et al.*, 2007b), however correlating enzyme activity was not quantified.

IL-1Ra knockout mice spontaneously develop degenerative IVD changes, compared to their wild type counterparts (Phillips *et al.*, 2013). This indicates that the balance between IL-1 agonist and antagonist expression is important for maintaining the health of the IVD.

1.4.5 Other Cytokines in the IVD

1.4.5.1 Interleukins

Whilst attention has been given to IL-1, more than 20 other interleukin series cytokines exist. Some, including IL-4, IL-6, IL-10, IL-12, IL-17 and IL-20 have been identified within the IVD. With the exception of IL-6 (described below), their role in IVD cell biology is largely unknown, Table 1-3 summarises current knowledge.

1.4.5.2 Interleukin-6

IL-6 is a secreted cytokine produced by many cell types. The IL-6 gene product varies in molecular weight from 21 to 28kDa dependent on post-translational modification and has diverse biological activity (Kishimoto *et al.*, 1995). Depending on the target cell type, IL-6 can induce cellular proliferation or differentiation and activate the innate immune system (Baumann & Gauldie, 1994; Lenczowski *et al.*, 1999). IL-6 and can also modulate the activity of other cytokines, in certain situations by down-regulating both IL-1 and TNF-α (Dinarello & Moldawer, 2000).

Transduction of the IL-6 signal is dependent on the interaction of IL-6/IL-6 receptor (IL-6R) complex with the membrane bound G-protein coupled signal transducing protein, gp130. This is a common signal transduction pathway shared with several other cytokines including IL-11, oncostatin-M (OSM) and leukaemia inhibitory factor (LIF) that show structural homology and related biological functions to IL-6 (Taga & Kishimoto, 1997).

IL-6 is expressed by native IVD cells (Kang *et al.*, 1996; Specchia *et al.*, 2002; Lee *et al.*, 2009) however, receptor expression has not been confirmed. The only study to investigate IL-6R expression in the human IVD indicates that the receptor is not expressed in normal IVDs, but that receptor expression is spontaneously induced in prolapsed tissue (Specchia *et al.*, 2002).

	Details	References
IL-1	↑ in degenerate and prolapsed IVDs *In vitro*: ↑ IL-6 synthesis, ↓ type II collagen and aggrecan transcription, ↓ proteoglycan synthesis, ↑ MMP synthesis, ↑ ADAMTS synthesis	(Doita *et al.*, 1996; Ahn *et al.*, 2002; Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d; Lee *et al.*, 2009; Studer *et al.*, 2011)
IL-4	Identified in degenerate and prolapsed IVDs	(Shamji *et al.*, 2010)
IL-6	Identified in non-degenerate, degenerate and prolapsed IVDs *In vitro*: ↓ type II collagen and aggrecan transcription, ↓ proteoglycan synthesis, ↑ MMP synthesis, ↑ PGE-2	(Kang *et al.*, 1996; Specchia *et al.*, 2002; Lee *et al.*, 2009; Shamji *et al.*, 2010; Studer *et al.*, 2011)
IL-10	Identified in prolapsed IVDs	(Ahn *et al.*, 2002)
IL-12	Identified in degenerate and prolapsed IVDs	(Lee *et al.*, 2009; Shamji *et al.*, 2010)
IL-17	Identified in degenerate and prolapsed IVDs *In vitro*: ↑ IL-6 synthesis, ↑ NO production, ↑ PGE-2 production,	(Shamji *et al.*, 2010; Gabr *et al.*, 2011)
IL-20	Identified in prolapsed IVDs alongside receptors *In vitro*: ↑ transcription of IL-1β, IL-6, CCL2 and MMP-3	(Huang *et al.*, 2008)

Table 1-3 Interleukin Family Cytokines, and Known Biological Activities, in the Human Intervertebral Disc

↑, increased; ↓, decreased; PGE-2, prostaglandin E2; NO, nitric oxide

To determine potential *in vitro* responses of human NP cells (related to anabolic and catabolic metabolism) to IL-6 stimulation, soluble IL-6R was also supplemented (which may bind the ligand prior to signalling through gp130) (Studer *et al.*, 2011). This study revealed that IL-6 stimulation affected anabolic metabolism by suppressing type II collagen and aggrecan transcription and affected catabolic metabolism by increasing MMP-3 translation (Studer *et al.*, 2011). The effects of stimulation on type I collagen, other MMPs or ADAMTSs were not investigated. Co-stimulation of IL-6 and soluble IL-6R with either IL-1 or TNF-α was seen to potentiate the effects of IL-1 or TNF-α stimulation alone, on all anabolic and catabolic parameters measured (Studer *et al.*, 2011). Stimulation with either IL-1, TNF-α or IL-6 and soluble IL-6R increased transcription of IL-6, with IL-1 inducing the most potent response (Studer *et al.*, 2011). The effect of IL-6 stimulation on transcription of IL-1 or TNF-α were not investigated however, the potentiating effects of co-stimulation indicate that in this experimental system, IL-6 stimulation does not have a down-regulatory effect on either IL-1 or TNF-α expression. These studies were performed on human NP cells derived from degenerate tissue, as such the responses described may be altered from those in NP cells derived from normal IVDs.

1.4.5.3 The Tumour Necrosis Factor Superfamily

The TNF superfamily has at least 19 members including TNF-α, TNF-β (lymphotoxin-α), Fas Ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), TNF-like weak inducer of apoptosis (TWEAK) and CD40 ligand (CD40L) (Gruss & Dower, 1995; Bazzoni & Beutler, 1996). With the exception of TNF-β, members are homotrimeric proteins presented on the extracellular plasma membrane (Bazzoni & Beutler, 1996). Biological similarities between members exist and most function to regulate cellular proliferation and apoptosis, some are also involved in activation of the innate immune system (Dinarello & Moldawer, 2000).

The rate of apoptosis is increased in the degenerate IVD compared to that of the normal (Tschoeke *et al.*, 2008; Wang *et al.*, 2011). Since TNF superfamily members are able to trigger apoptosis, several investigations have studied their expression in the IVD.

Apoptosis, or programmed cell death, is in most cases a normal cellular event (Mera, 1997). Cells that have reached the end of their useful lives undergo apoptosis and are replaced by division of neighbouring cells so that a balance of tissue cellularity is maintained (Mera, 1997). The process is mediated by cysteinyl-

aspartate-specific proteases (caspases) that instigate the cell shrinkage and DNA fragmentation characteristic of apoptosis (Hancock, 2005). Intrinsic or extrinsic signalling pathways may activate caspases and trigger apoptosis.

The intrinsic pathway is activated in response to DNA damage or cellular stress and results in increased permeability of mitochondria and release of mitochondrial cytochrome-*c* into the cytosol (Antonsson, 2004). Cytochrome-*c* then complexes with other pro-apoptotic factors to form the apoptosome (Antonsson, 2004). The extrinsic pathway is initiated by ligand binding to extracellular membrane presented death receptors (DRs) (Wang & El-Deiry, 2003). The intracellular C-terminal of DRs contains a 'death domain' through which the signal is transduced to intracellular pro-apoptotic factors that form the death-induced signalling complex (Wang & El-Deiry, 2003). The apoptosome and death-induced signalling complex contain precursor initiator caspase molecules that are activated by autocatalytic cleavage. These in turn activate effector caspases and apoptosis results.

Within the IVD, caspases specific to each signalling pathway have been investigated. Caspase-9, an initiator caspase in the intrinsic pathway, is increased in tissue homogenate from degenerate samples compared to normal (Tschoeke *et al.*, 2008; Wang *et al.*, 2011), as is caspase-8, an initiator caspase in the extrinsic pathway (Tschoeke *et al.*, 2008; Wang *et al.*, 2011). Expression of both caspases correlates positively with increasing severity of degenerative tissue changes, up to moderate levels after which no further increase occurs (Wang *et al.*, 2011). Effector caspase activity (caspase -3 and -7) (Tschoeke *et al.*, 2008), alongside rate of apoptosis (Tschoeke *et al.*, 2008; Wang *et al.*, 2011), is also increased and correlates positively with the severity of degenerative tissue changes (Wang *et al.*, 2011). These findings indicate that increased apoptosis in the degenerate IVD is by up-regulation of both intrinsic and extrinsic pathways.

Several pro-apoptotic TNF superfamily members may mediate apoptosis via the extrinsic pathway, including FasL and TNF-α. Transduction of the FasL signal is dependent on interaction with Fas receptor, and transduction of an apoptotic signal by TNF-α is dependent on ligand interaction with TNF-RI (TNF-R55), both of which possess intracellular 'death domains' that may signal for death-induced signalling complex formation.

FasL (Park *et al.*, 2001a) and TNF-α are expressed by native IVD cells, as are Fas receptor (Park *et al.*, 2001b) and TNF-RI. Transcription of both FasL and Fas receptor is increased in degenerate tissue homogenate compared to non-degenerate (Tschoeke *et al.*, 2008; Wang *et al.*, 2011). Observed transcription

patterns are similar to that of caspase-8 activity, with transcription increasing concordant to severity of degenerative tissue changes up to moderate levels without a further increase between moderately and severely degenerate tissues (Wang *et al.*, 2011). As previously described, TNF-α expression is up-regulated in degenerate IVDs however, DR, TNF-RI, expression is unaffected.

These findings indicate that the extrinsic pathway is, at least in part, responsible for the increased rates of apoptosis observed in IVD degeneration. Excessive or dysregulated cytokine expression may drive this process through DR signalling. Apoptosis of native cells may also form part of the mechanism of spontaneous resorption of prolapsed IVD tissue. FasL and Fas receptor transcription are increased in prolapsed IVD tissue compared to normal, as is caspase activity (Wang *et al.*, 2011). Further, extruded and sequestered tissue samples reveal greater expression and activity rates indicating that the more severe the degree of prolapse the greater the apoptotic response generated (Wang *et al.*, 2011). This mechanism may account in part for the destruction of displaced IVD tissue.

Other roles for TNF superfamily members within the IVD have been postulated. Nerve growth factor (NGF) is produced by native IVD cells (Freemont *et al.*, 1997; Freemont *et al.*, 2002; Richardson *et al.*, 2009; Lee *et al.*, 2011) and may promote the innervation seen in severely degenerate IVDs (Lee *et al.*, 2011).

1.4.5.4 Chemokines

The chemokines (chemo-attractive cytokines) are at least 50 small (8 - 10 kDa) secreted protein molecules, split between four major families based on structural similarity (Figure 1-10). The tertiary structure of all chemokines is similar and accounted for by disulphide bonds that form between conserved cysteine residues (Clark-Lewis *et al.*, 1995). This structural similarity conveys characteristic chemokine properties. Signal sequestration occurs so that chemokines become bound to GAG molecules on the cell surface or ECM via ionic interaction of basic residues in the C-terminal helix and core region (Chakravarty *et al.*, 1998; Amara *et al.*, 1999). Bound chemokines retain their full biologic activity (Webb *et al.*, 1993; Middleton *et al.*, 1997) and receptor interaction is through the N-terminal domain and exposed 'loop region' that follows the second conserved cysteine residue (Thelen, 2001). This property explains the locally focused response elicited by chemokine secretion.

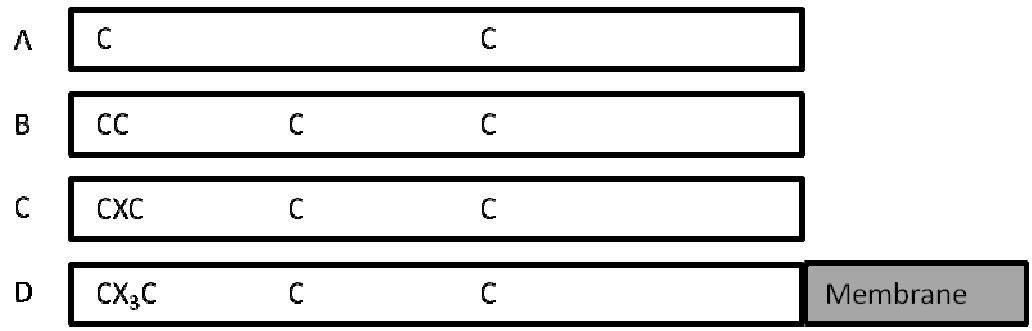


Figure 1-10 Schematic Representation of Chemokine Structural Subfamilies

Four chemokine structural subfamilies exist based on the positioning of conserved cysteine residues within the N-terminal motif; C = cysteine residue; X = other amino acid residue. XC chemokines (A) have only one N-terminal cysteine residue e.g. XCL1. C-C chemokines (B) have two adjacent conserved cysteine residues, e.g. CCL3. C-X-C chemokines (C) have two conserved cysteine residues separated by one other amino acid residue, e.g. CXCL8. C-X_3-C chemokines (D) have two conserved cysteine residues separated by three other amino acids, the only identified member of this structural subfamily, CX_3CL1, has a C-terminal membrane-association domain that accounts for its anchoring to the plasma membrane.

Table 1-4 Cytokines and Chemokines in the Intervertebral Disc, and those implicated in the Pathogenesis of Osteoarthritis and Rheumatoid Arthritis

Cytokines and Chemokines listed are those identified in the IVD and/or those implicated in the pathogenesis of osteoarthritis (OA) or rheumatoid arthritis (RA). This list is not exhaustive in respect of OA and RA. Chemokine systematic nomenclature is used – alternative names are given in Appendix 5.

	IVD	References	OA	References	RA	References
Cytokines						
IL-1	✔	(Doita *et al.*, 1996; Ahn *et al.*, 2002; Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d; Lee *et al.*, 2009; Studer *et al.*, 2011)	✔	(Moo *et al.*, 2001; David *et al.*, 2007; Sandell *et al.*, 2008)	✔	(Andreas *et al.*, 2008; Weiss *et al.*, 2009)
IL-4	✔	(Shamji *et al.*, 2010)	✔	(Moo *et al.*, 2001)		
IL-6	✔	(Kang *et al.*, 1996; Specchia *et al.*, 2002; Lee *et al.*, 2009; Shamji *et al.*, 2010; Studer *et al.*, 2011)	✔	(Moo *et al.*, 2001; Pulai *et al.*, 2005; David *et al.*, 2007; Sandell *et al.*, 2008)	✔	(Pulai *et al.*, 2005; Andreas *et al.*, 2008; Kelchtermans *et al.*, 2009)
IL-7					✔	(Borzi *et al.*, 2000; Dinarello & Moldawer, 2000)
IL-10	✔	(Ahn *et al.*, 2002)	✔	(Moo *et al.*, 2001; Muller *et al.*, 2008)	✔	(Moo *et al.*, 2001; Muller *et al.*, 2008)
IL-11					✔	(Nagineni *et al.*, 2009)
IL-12	✔	(Lee *et al.*, 2009; Shamji *et al.*, 2010)				
IL-15					✔	(Woolley & Tetlow, 2000)
IL-16					✔	(Dinarello & Moldawer, 2000)
IL-17	✔	(Shamji *et al.*, 2010; Gabr *et al.*,	✔	(Abramson & Yazici, 2006)	✔	(Van Bezooijen *et al.*, 1999; Kelchtermans *et al.*, 2009; Moran

	IVD	References	OA	References	RA	References
		2011)				*et al.*, 2009)
IL-18			✔	(Abramson & Yazici, 2006)		
IL-20	✔	(Huang *et al.*, 2008)				
IL-23					✔	(Andreas *et al.*, 2008)
TNF-α	✔	(Ahn *et al.*, 2002; Weiler *et al.*, 2005; Bachmeier *et al.*, 2007; Jee *et al.*, 2007; Le Maitre *et al.*, 2007d; Hoyland *et al.*, 2008)	✔	(Moo *et al.*, 2001)	✔	(Andreas *et al.*, 2008; Moran *et al.*, 2009)
CSF2	✔	(Weiler *et al.*, 2005)			✔	(Kelchtermans *et al.*, 2009)
CSF3			✔	(Sandell *et al.*, 2008)	✔	(Andreas *et al.*, 2008; Sandell *et al.*, 2008)
IFN-γ			✔	(Moo *et al.*, 2001)	✔	(Moo *et al.*, 2001; Kelchtermans *et al.*, 2009)
OSM					✔	(Moran *et al.*, 2009)
LIF			✔	(Sandell *et al.*, 2008)		
Chemokines						
CCL2	✔	(Kikuchi *et al.*, 1998; Burke *et al.*, 2002; Ohba *et al.*, 2008)	✔	(Yuan *et al.*, 2001; Yoshida *et al.*, 2002; Silvestri *et al.*, 2003; Pulai *et al.*, 2005)	✔	(Pulai *et al.*, 2005; Andreas *et al.*, 2008)
CCL3	✔	(Wang *et al.*, 2012)	✔	(Borzi *et al.*, 1999; Yuan *et al.*, 2001; Silvestri *et al.*, 2003; Sandell *et al.*, 2008)	✔	(Borzi *et al.*, 1999)

Table 1-4 Continued from previous page.

	IVD	References	OA	References	RA	References
CCL4	✔	(Wang *et al.*, 2012)	✔	(Borzi *et al.*, 1999; Yuan *et al.*, 2001; Sandell *et al.*, 2008)	✔	(Borzi *et al.*, 1999; Andreas *et al.*, 2008)
CCL5	✔	(Ahn *et al.*, 2002; Kawaguchi *et al.*, 2002)	✔	(Yuan *et al.*, 2001; Silvestri *et al.*, 2003)		
CCL7	✔	(Kawaguchi *et al.*, 2002)				
CCL8			✔	(Sandell *et al.*, 2008)		
CCL13	✔	(Kawaguchi *et al.*, 2002)				
CCL20			✔	(Sandell *et al.*, 2008)		
CXCL1		(Kawaguchi *et al.*, 2002)	✔	(Borzi *et al.*, 1999; Merz *et al.*, 2003; Silvestri *et al.*, 2003; Sandell *et al.*, 2008)	✔	(Borzi *et al.*, 1999; Andreas *et al.*, 2008)
CXCL2			✔	(Pulai *et al.*, 2005; Sandell *et al.*, 2008)		
CXCL3			✔	(Sandell *et al.*, 2008)	✔	(Andreas *et al.*, 2008)
CXCL5			✔	(Sandell *et al.*, 2008)		
CXCL6					✔	(Kelchtermans *et al.*, 2009)
CXCL8	✔	(Ahn *et al.*, 2002; Burke *et al.*, 2002; Jee *et al.*, 2007)	✔	(Borzi *et al.*, 1999; Brisby *et al.*, 2002; Merz *et al.*, 2003; Silvestri *et al.*, 2003; Pulai *et al.*, 2005; David *et al.*, 2007; Sandell *et al.*, 2008)	✔	(Borzi *et al.*, 1999; Pulai *et al.*, 2005; Andreas *et al.*, 2008)
CXCL9	✔	(Kawaguchi *et al.*, 2002)				

Table 1-4 Continued from previous page.

	IVD	References	OA	References	RA	References
CXCL10	✔	(Kawaguchi *et al.*, 2002)				
CX_3CL1			✔	(Sandell *et al.*, 2008)		

Table 1-4 Continued from previous page.

The biological activities of chemokines are diverse however, all possess the common property of being able to induce chemotaxis, which is the selective directional migration of target cells. As such chemokines are closely associated with the innate and adaptive immune responses. They function to recruit leukocytes to sites of infection, injury and disturbed tissue homeostasis as well as modulating lymphocyte differentiation and activation in haematopoiesis (Moser *et al.*, 2006). Chemokines may also function to promote or dampen inflammatory responses (Wakugawa *et al.*, 2001; Eddleston *et al.*, 2002), neovascularisation (Romagnani *et al.*, 2006), or pain sensation (Zhang & Oppenheim, 2006). They stimulate superoxide production (Huber *et al.*, 2002), protease release (Cross & Woodroofe, 1999), generation of reactive lipid products, phenotypic and cytoskeletal changes (Banas *et al.*, 2004), proliferation (Bonacchi *et al.*, 2001) and modulation of ECM synthesis (Banas *et al.*, 1999; Romagnani *et al.*, 1999; Schecter *et al.*, 2000).

Chemokine expression within the prolapsed IVD has been investigated, since expression may modulate the observed infiltration of inflammatory cells, therefore forming part of the mechanism of spontaneous resorption. To this end several chemokines have been identified as expressed by native IVD cells within prolapsed tissue (Table 1-4). For the majority of these chemokines it remains unknown whether their expression is a spontaneous response to tissue displacement or an event that occurs from the normal physiological conditions to which the native cells are exposed. The only studies to use normal IVD tissue alongside prolapsed, indicated that CCL2, CCL3, CCL4 and CXCL8 expression is evident in normal IVDs, albeit increased in prolapsed tissue (Burke *et al.*, 2002; Wang *et al.*, 2012).

1.4.5.5 Chemokine Intracellular Signalling

Responses to chemokine signalling are generated through ligand binding to seven transmembrane trimeric G-protein coupled receptors (Thelen, 2001). Chemokine receptors are split into families based on the N-terminal motif of the chemokines that they bind. Most receptors recognise more than one chemokine and several chemokines bind to more than one receptor (Murphy *et al.*, 2000).

Ligand binding promotes conformational changes in the receptor, such that the α subunit is dissociated from the βγ subunit, dependent on the exchange of GDP for GTP (Thelen, 2001). Dependent on the ligand that is bound, conformational changes in the receptor may result in generation of diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP3). IP3 binding to its receptor at the endoplasmic reticulum triggers the release of intracellular calcium (Kiselyov *et al.*, 2003).

Increased intracellular calcium alongside the presence of DAG causes activation of protein kinase C (PKC) and signalling through the phosphoinositide-specific phospholipase C (PLC) pathway (Kiselyov et al., 2003). Receptor conformational changes may also activate the PI3K signalling pathway (Bonacchi *et al*., 2001) (described previously (section 1.4.1.1)).

1.4.6 Cytokines and Chemokines in Other Arthropathies

The roles of cytokines and chemokines in osteoarthritis (OA) and rheumatoid arthritis (RA) are well documented. IL-1 and TNF-α are considered primary effectors capable of inducing other cytokines and chemokines regarded as second step mediators of disease (Van den Berg & Miossec, 2004). Cytokines and chemokines implicated in the pathology of OA and RA are summarised in Table 1-4.

1.5 Low Back Pain

The anatomic source of low back pain (LBP) is difficult to specify (Hancock *et al.*, 2007) and can arise from several or any combination of multiple sources. The innervated structures of the vertebral bodies, facet joints, sacroiliac joints and local ligaments and musculature of the motion segment are all implicated (Cavanaugh *et al.*, 1996; Hancock *et al.*, 2007). IVD degeneration increases the risk of LBP development (Luoma *et al.*, 2000) and studies indicate that 40% of LBP cases are linked directly to IVD degeneration (Schwarzer *et al.*, 1995) through direct mechanical and/or peripheral biochemical stimulation of local nerves.

The application of force (tension, torsion, compression or pressure) abnormal to that physiologically expected, to an innervated structure may generate a pain signal propagated by local mechanoreceptors (Cavanaugh *et al.*, 1996). The altered biomechanics of a motion segment that contains a degenerate IVD, results in abnormal loading of tissue structures during normal movement (Figure 1-11). The lumbar IVD becomes a further source of LBP when structural disruption is sufficient to result in abnormal loading of the innervated outer AF. Progressively, neo-innervation of the IVD occurs with enhanced nociceptive (pain sensing) nerve fibre penetration (Freemont *et al.*, 1997; Freemont *et al.*, 2002). Therefore, whilst the progressive biomechanical failure of the IVD enhances generation of mechanical pain stimuli, the IVD itself becomes hyper-sensitised to pain detection.

Biochemical factors may also enhance the propagation of pain signals. Local concentrations of cytokines (IL-1, TNF-α, IL-6), prostaglandins, phospholipases and neuropeptides (substance P) propagate pain signals through receptor-mediated

Image removed for copyright reasons

Figure 1-11 Schematic Representation of the Anatomical Sources of Low Back Pain

Low back pain can arise from many or any combination of multiple, anatomical sources. The innervated structures of the vertebral bodies, facet joints, sacroiliac joints and local ligaments and musculature of the motion segment are all implicated. The lumbar IVD may also be a source of low back pain if degenerative changes are sufficient to result in abnormal loading patterns through the motion segment. Figure adapted from Posavec & Saftic (2010).

binding to local nerve cells (Watkins *et al.*, 1995). This mechanism may further enhance painful stimulation of nerve endings within the IVD.

Prolapse of the IVD may result in LBP from direct compression of adjacent nerve roots. However, direct compression is not required since prolapse that does not result in compression of nerve roots is also seen to induce LBP (Mulleman *et al.*, 2006a; Mulleman *et al.*, 2006b). Biochemical irritation of adjacent nerve roots is thought to account for painful stimulation in these cases.

1.6 Conclusions

LBP is a prevalent, disabling condition that represents a significant economic burden in many Western industrialised societies (Maniadakis & Gray, 2000; Dagenais *et al.*, 2008). Progressing the development of effective treatment strategies can only arise from a greater understanding of the complex, multi-factorial processes of the pathogenesis of LBP.

Cytokines may represent a critical mechanism in the development of LBP, particularly related to IVD structural failure and biochemically mediated local hyperalgesic states. Improvement in understanding in this area may lead to more effective treatment strategies by identifying potential therapeutic targets. Currently, several research initiatives are investigating the regenerative potential of stem cell therapy in the IVD (Steck *et al.*, 2005; Le Maitre *et al.*, 2009; Collin *et al.*, 2011; Yang *et al.*, 2011). To this end, an understanding of the micro-environmental conditions within the 'degenerate niche' would provide insights as to the effects that may be elicited on the behaviour of cells introduced to such an environment.

1.7 Aims and Objectives

The purpose of these studies is to investigate the hypothesis that;

Cytokines and chemokines are integral to the pathogenesis of IVD degeneration and prolapse that may lead to the generation of LBP.

Specifically;

- To identify cytokines and chemokines produced within the NP of normal, degenerate and prolapsed IVDs
- To identify the 'target cells' of these cytokines and chemokines based on receptor expression
- To determine the inter-relationship that exists between these cytokines and chemokines within the IVD
- To understand how the different expression patterns and inter-relationships may contribute to disruption of catabolic and anabolic cellular metabolism

2 Materials and Methods

2.1 Tissue Sample Processing

Human IVD tissue was obtained either at post-mortem (PM) examination or surgery with informed consent of the patient or relatives. Details of all IVD tissue samples used in these studies are shown in Appendix 1. Post-mortem (PM) IVD tissue samples were supplied by Leeds Institute of Molecular Medicine Tissue Bank. Research Ethics Committee approval to use human tissue taken at the time of spinal surgery was obtained from Sheffield Research Ethics Committee - REC 09/H1308/70.

2.1.1 Sample Collection

2.1.1.1 Surgical Intervertebral Disc Tissue

IVD tissue samples were obtained at surgery from patients undergoing micro-discectomy procedures at the Northern General Hospital, Sheffield, for the treatment of sciatica and nerve compression.

Upon removal IVD tissue was placed into sterile 50mL collection tubes containing 20mL Serum Free Media (SFM; Dulbecco's Modified Eagles Media (DMEM; Gibco) supplemented with 200U/mL penicillin (Gibco), 200µg/mL streptomycin (Gibco), 500ng/mL amphotericin B (Sigma), 50µg/mL ascorbic acid (Sigma) and 2mM L-glutamine (Gibco)) and kept at 4°C for collection and transportation to the laboratory for processing. Tissue samples were accompanied by a Disc Material Details form (Appendix 2) completed by the surgical team at the time of sample collection.

2.1.1.2 Post-Mortem Intervertebral Disc Tissue

PM tissue samples consisted of intact IVDs within the complete motion segment. Upon recovery, motion segments were placed into sterile 500mL collection vessels containing 200mL SFM and kept at 4°C for collection and transportation to the laboratory for processing.

2.1.2 Sample Processing

2.1.2.1 Surgical Intervertebral Disc Tissue

Surgical samples consisted of multiple fragments of IVD tissue. The majority of tissue fragments were NP however some were NP with CEP and/or AF attached. The tissue samples were examined and used either for embedding to paraffin wax as detailed in section 2.1.3, or to be split between embedding to wax and NP cell isolation. In the case of the latter, fragments were examined and separated, tissue with CEP and/or AF attached was placed for embedding in all cases, only NP tissue was used for cell isolation as detailed in section 2.1.4.

2.1.2.2 Post-Mortem Intervertebral Disc Tissue

In the laboratory, PM IVDs were dissected from within the motion segment. From whole IVDs, blocks of tissue comprising NP and AF in continuity were cut and set for embedding to paraffin wax. Remaining NP tissue was used for isolation of NP cells.

2.1.3 Embedding to Paraffin Wax

Tissue samples were fixed immediately by immersion in 10% Neutral Buffered Formalin at 4°C for between 48 and 72 hours. Following fixation samples were processed to paraffin wax on a Shandon Elliott Duplex Processor in an automated process of dehydration by immersion in alcohol (IMS; Industrial Methylated Spirit, Fisher Scientific) and clearing in Sub-X (Xylene Substitute, Leica) prior to immersion in molten paraffin wax, full processing details are given in Appendix 3. Samples in molten wax were transferred to a JeioTech OV-11 vacuum oven and incubated at 60°C and 60cmHg for 1 hour to ensure complete penetration of wax throughout the sample. Tissue samples were then oriented as desired into moulds containing molten wax and allowed to cool into blocks. Once set tissue blocks were removed from moulds and stored at room temperature to be used as described in sections 2.2 and 2.3 for histology and immunohistochemistry.

2.1.4 Isolation of Nucleus Pulposus Cells

NP tissue was finely dissected and washed with 2U/mL protease (Sigma) in SFM for 30 minutes at 37°C, to lyse any blood cells contaminating the tissue surface. Tissue was washed twice in SFM and cells released by digestion of ECM in 2mg/mL Collagenase type I (Sigma) in SFM for between 4 and 18 hours at 37°C rotating gently on a flatbed shaker. The cell suspension was filtered through sterile 40µm cell strainers (Beckton Dickinson) and cells recovered by centrifugation at 400g for 10 minutes. The resulting cell pellet was washed twice by re-suspension in SFM and centrifugation before being re-suspended in SFM. Isolated cells ('directly extracted'; (DE)) were used immediately for total RNA extraction and/or cell culture as detailed in sections 2.1.5 and 2.4.

2.1.5 Total RNA Extraction from Directly Extracted Nucleus Pulposus Cells

Isolated NP cells were counted using an Invitrogen Countess automated cell counter (Invitrogen) incorporating trypan blue (Gibco) to distinguish live and dead cells. Two cell suspension aliquots containing 1.0 x 10^5 live cells were taken to RNase free 1.5mL tubes and centrifuged at 400g for 10 minutes to recover cells. Supernatant was removed and the remaining cell pellet re-suspended in 1mL Trizol (Ambion) and incubated at room temperature for 5 minutes. 200µL Chloroform

(Sigma) was added per tube; samples were mixed by vortexing for 15 seconds and incubated at room temperature for 3 minutes prior to centrifugation at 12000g for 15 minutes at 4°C. Following centrifugation, the upper aqueous phase (RNA containing) was removed to a fresh 1.5mL tube and 500µL isopropanol (Sigma) added. RNA samples were incubated for 10 minutes at room temperature and then at -80°C for 1 hour to precipitate RNA. Samples were thawed on ice and centrifuged at 12000g for 30 minutes at 4°C to collect RNA precipitate. The resulting pellet was re-suspended in 87.5µL sterile deionised water. Samples were subjected to DNase digestion to remove any remaining traces of genomic DNA by addition of 12.5µL DNase solution containing 30U DNase I (Qiagen; RNase-Free DNase Kit) and incubation for 10 minutes at room temperature. RNA was recovered and DNase removed using Qiagen Min-Elute Clean-Up Kit as per manufacturer's protocol. RNA samples were eluted in 14µL sterile deionised water and used immediately for cDNA synthesis as described in section 2.5.2. Cell suspension remaining following collection of aliquots for total RNA extraction was used in cell culture as described in section 2.4.

2.2 Histology

All formalin fixed paraffin embedded (FFPE) tissue samples were subjected to histological assessment to determine the grade of tissue degeneration and the presence or absence of 'infiltrating' cells.

2.2.1 Sectioning and Mounting

Tissue sections were cut using a Leica SM2400 sledge microtome to 4µm thickness. Tissue sections were placed onto the surface of a 45˚C water bath and mounted by floating onto positively charged adhesive slides (Leica). Excess water was dried from slides and sections by 30 minute incubation on a 40˚C drying rack. Mounted sections were then transferred into a desiccating oven at 37˚C to complete drying and for storage until use.

2.2.2 Haematoxylin and Eosin Staining

Sections were de-waxed by immersion in Sub-X (Leica) for 5 minutes in triplicate followed by rehydration in IMS (Fisher Scientific) for 5 minutes in triplicate. Sections were incubated in Mayer's Haematoxylin (Leica) for 60 seconds then 'blued' by immersion in running water for 5 minutes prior to incubation in Eosin Y (Leica) for 60 seconds. Sections were dehydrated by immersion in IMS for 3 minutes in triplicate followed by clearing in Sub-X for 5 minutes in triplicate. Sections were mounted by application of 2 drops Pertex mountant (Leica) per tissue section and placement of 1µm thickness glass cover slips.

2.2.3 Grading of Tissue Sections

Tissue sections were evaluated using an Olympus BX60 research microscope. Each section was assigned a grade between 0 and 12 based on the presence of histological features associated with IVD degeneration pathology. A score of 0 - 3 indicates a histologically normal (non-degenerate) IVD and a grade of 4 - 12 indicates evidence of degeneration. All sections were graded independently by two observers (KLEP and CLLM), the grade assigned being the average of the two observers' scores. Where deviations of greater than 2 grades were recorded between observers, sections were re-evaluated by both observers.

The grading system used was developed by Professor Antony Freemont (Histopathologist, University of Manchester) and Dr Christine Le Maitre, and applied as previously published (Le Maitre *et al.*, 2005). Sections were scored for the presence of cell clusters, the presence of fissures, the loss of demarcation between AF and NP and the loss of proteoglycan within the NP based on haematoxyphilia. In surgical tissue samples, it was often impossible to score the loss of demarcation since AF and NP in continuity was rarely obtained. Grades were assigned to these samples by multiplying the sum of scores from the other 3 factors by 0.75. Table 2-1 gives full details of the grading system used and image illustrations are shown in Figure 2-1.

2.2.3.1 Infiltrated Tissue Samples

Tissue sections were also evaluated for the presence of 'infiltrating' cells within the NP. Infiltrating cells were identified based on cell morphology – namely an elongated shape and an absence of surrounding lacunae as shown in Figure 2-2.

2.2.4 Classification of Tissue Samples

2.2.4.1 qPCR Studies

For qPCR studies cDNA derived from tissue samples was classified into non-degenerate, degenerate or infiltrated study groups based on histological evaluation of matched FFPE tissue. Multiple sections from each paraffin block were subjected to histological evaluation. Matched cDNA samples were classified 'degenerate' if any tissue section was graded greater than 4, and/or classified 'infiltrated' if infiltrating cells were observed on any tissue section.

2.2.4.2 Immunohistochemistry Studies

When FFPE tissue was sectioned for IHC studies adjacent serial sections were taken for histological assessment. Classification into non-degenerate, degenerate and infiltrated study groups was based only on assessment of the IHC section and adjacent histology section. Therefore sections are only classified 'degenerate' if

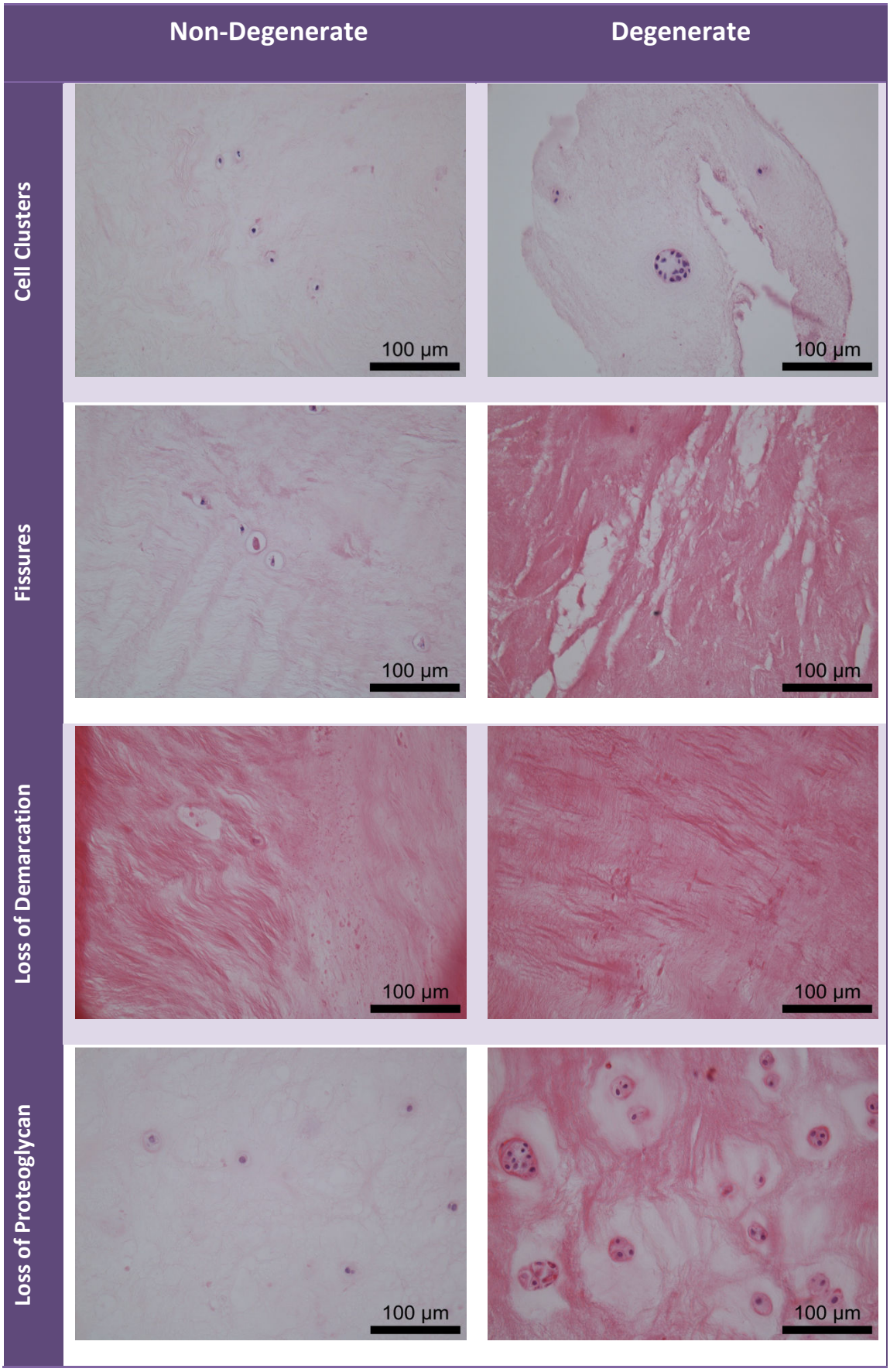
Histological Features;		Score
Presence of Cell Clusters	Cell clusters not present	0
	<25% Cells present in clusters	1
	25% - 75% cells present in clusters	2
	>75% cells present in clusters	3
Presence and Extent of Fissures	Fissures not present	0
	Fissures present within NP	1
	Fissures extending to junction of NP and AF	2
	Fissures extending into AF	3
Loss of Demarcation between AF and NP	Clear demarcation	0
	Limited loss of demarcation	1
	Substantial loss of demarcation	2
	Complete loss of demarcation	3
Loss of Proteoglycan within NP	No loss of haematoxyphilia	0
	Limited loss of haematoxyphilia	1
	Substantial loss of haematoxyphilia	2
	Complete loss of haematoxyphilia	3

Table 2-1 Histological Grading System used to Score Degeneration in Tissue Sections

All sections were evaluated independently by two researchers (KLEP and CLLM). The grade assigned was the mean of the two observers' scores.

Figure 2-1 Images Showing Characteristic Histological Features of Intervertebral Disc Degeneration as Assessed to Determine Grade of Degeneration in Nucleus Pulposus Tissue Sections

Non-Degenerate, top to bottom: In non-degenerate IVDs NP cells are found within distinct lacuna as single or doublet cells (HD89), fissures within the NP are not observed (HD52); there is clear demarcation between AF and NP (HD30) and consistent proteoglycan content throughout the NP (HD53). Degenerate, top to bottom: In degenerate IVDs clusters of cells within the same lacuna are commonly observed (HD3), fissures form within the NP that can extend into the AF (HD40), demarcation between the AF and NP is lost as the NP becomes more fibrous (HD41) and proteoglycan depletion becomes visible in the peri-cellular region (HD15). Full details of samples (HD) are given in Appendix 1.


Non-Degenerate
Degenerate
Cell Clusters
Fissures
Loss of Demarcation
Loss of Proteoglycan
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm

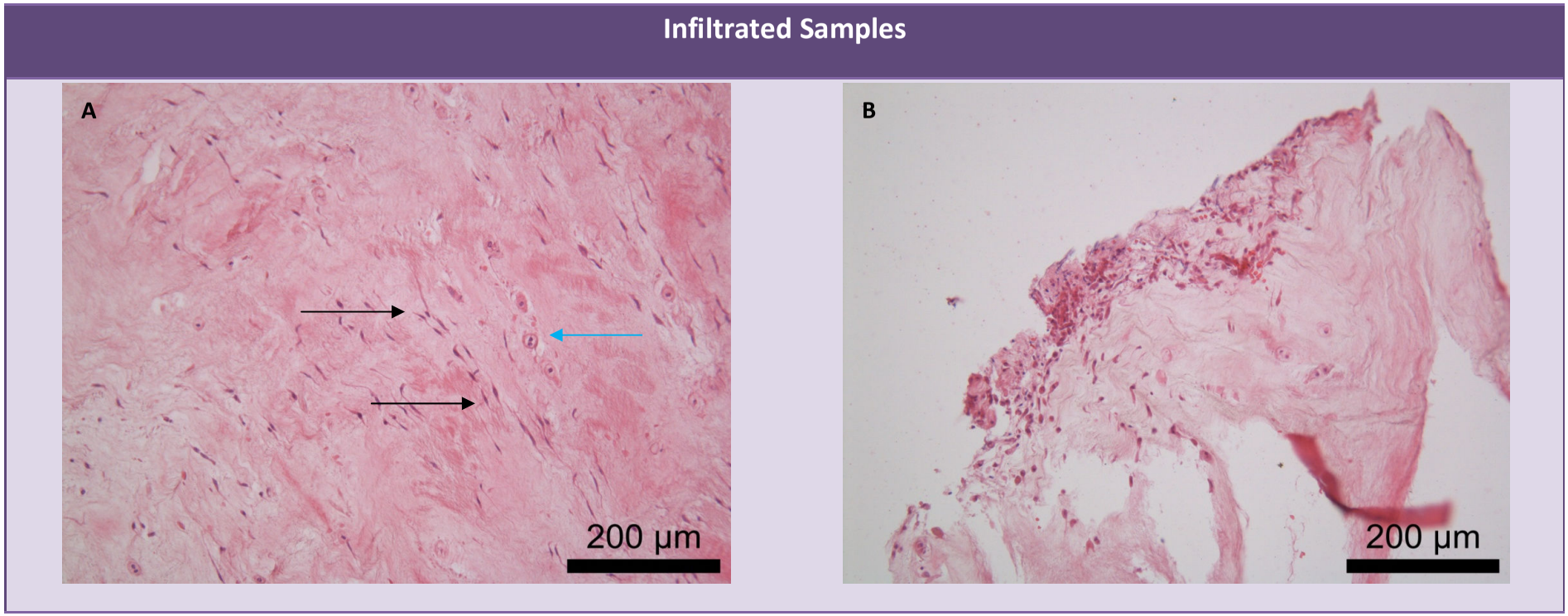


Figure 2-2 Images Showing Characteristic Histological Identification of Infiltrated Nucleus Pulposus Tissue Sections as Assessed to Determine Sample Classification

'Infiltrating' cells were identified by cell morphology (A); an elongated shape and absence of surrounding lacuna as indicated by black arrows, in contrast NP cells visible in the same tissue section are rounded and within lacuna as indicated by blue arrow (HD36). (B) On occasions infiltrating cell masses were observed around the periphery of tissue sections (HD79). Full details of samples (HD) are given in Appendix 1.

signs of degeneration are present on the tissue section evaluated for IHC staining. Similarly sections are only classified 'infiltrated' if the presence of infiltrating cells is observable on the tissue section evaluated for IHC staining.

2.3 Immunohistochemistry

2.3.1 The Principle of Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to localise target antigens in tissue sections. A primary antibody specific to the antigen is applied to the tissue section and later a secondary antibody directed against a species-specific portion of the primary antibody is applied. The secondary antibody is conjugated, usually to a fluorochrome, enzyme or biotin, and consequently the location of the bound primary antibody can be visualised, depending on the label, by either fluorescence emission or detection of the products of an enzymatic reaction.

2.3.1.1 Streptavidin-Biotin-HRP DAB Method

In these investigations a streptavidin-biotin-HRP DAB method of IHC was used. This method was chosen based on evaluation of tissue sections by light microscopy, facilitating a simple means of comparison between tissue sections stained for IHC targets and for histological evaluation.

In this method, a biotin conjugated secondary antibody directed against the bound primary antibody is used to detect the target antigen (True, 2008). Following secondary antibody binding the enzymatic 'streptavidin-biotin' complex forms on addition of ABC reagent (horseradish peroxidase (HRP) labelled streptavidin solution), since the streptavidin protein binds biotin molecules with strong affinity (Yamashita, 2007). Visualisation is by enzymatic reaction, a dark brown precipitate forms at the site of secondary antibody binding following application of the chromagen, 3-3'-diaminobenzidine tetrahydrochloride (DAB), as a result of HRP activity (Yamashita, 2007). It is therefore important during the experimental protocol to neutralise endogenous peroxidases within tissue sections to limit non-specific visualisation. Finally, counterstaining with haematoxylin allows for determination of cellular localisation of IHC-staining in relation to cell nuclei (True, 2008).

2.3.2 Preparation of Tissue Sections

FFPE tissue samples were sectioned and mounted as described in section 2.2.1. All experimental procedures and incubations were performed at room temperature and all 'wash steps' were performed on a slow moving flatbed shaker for 5 minutes unless otherwise stated.

Sections in each batch were placed into slide racks and oriented so that primary antibody and isotype control sections from the same tissue block were adjacent. This minimised the effects of spatial separation particularly related to microwave irradiation and heat distribution during antigen retrieval.

Sections were de-waxed and rehydrated as previously described in section 2.2.2. Endogenous peroxidases were blocked by immersion in 3% v/v hydrogen peroxide (Sigma) in methanol (Fisher Scientific) for 30 minutes. Sections were washed once in deionised water then twice in Tris-Buffered Saline (TBS; 20mM Tris (Fisher Scientific), 150mM NaCl (Fisher Scientific), pH7.5). Antigen retrieval was performed immediately.

2.3.3 Antigen Retrieval

2.3.3.1 Optimisation

In order for successful antigen detection by immunohistochemical techniques antigens must be available for primary antibody binding. In their native conformation proteins may be bound to intra-, inter- or extra-cellular proteins making them unavailable for primary antibody binding (Pileri *et al.*, 1997). In addition to this, the chemical fixation and processing of tissues to paraffin, performed here to preserve morphology, catalyses further formation of molecular cross links that may mask antigens (Shi *et al.*, 2007).

Antigen Retrieval (AR) is the process of 'un-masking' these antigens and is routine for FFPE tissues. Many AR methods exist; most are based on either enzymatic digestion, treatment with protein denaturants or the application of heat (Kim *et al.*, 2004; Emoto *et al.*, 2005; D'Amico *et al.*, 2009).

In these studies several methods of enzymatic digestion and heat treatment were investigated to achieve successful AR (Table 2-2). Following these investigations a standard protocol of heat-induced AR by microwave irradiation in basic Tris-HCl buffer was adopted.

2.3.3.2 Standard Protocol

Sections were immersed in 400mL antigen retrieval buffer (0.05M Tris-HCl, pH 9.5) pre-heated to 60˚C and irradiated for 5 minutes in a Sanyo 800 watt microwave oven, set to deliver 40% of maximum power. Sections were cooled for 60 seconds at room temperature then subjected to another period of irradiation as described above. Sections were incubated in the hot buffer for 15 minutes at room temperature to allow cooling and re-folding of tissue antigens.

Method of Antigen Retrieval		Assessment
Enzymatic Digestion		
Chymotrypsin	Incubation in 0.01% chymotrypsin solution at 37°C for 30 minutes	Poor antigenicity of tissue sections
Hyaluronidase	Incubation in 0.1% hyaluronidase solution at 37°C for 30 minutes	Poor antigenicity of tissue sections
Heat Treatment		
Water Bath	Overnight incubation at 80°C in 0.05M Tris-HCl pH 9.5 AR buffer	Improved antigenicity, excessive damage to tissue sections
Microwave	10 minute microwave irradiation in 0.05M Tris-HCl pH 9.5 AR buffer	Improved antigenicity, little damage to tissue sections

Table 2-2 Enzymatic Digestion and Heat Treatment Methods of Antigen Retrieval Investigated to Establish Antigen Retrieval Standard Protocol

Four methods of antigen retrieval, commonly used to improve antigenicity in sections from formalin fixed paraffin embedded tissues were investigated. Based on microscopic assessment of sections subjected to antigen retrieval, heat treatment by microwave irradiation was adopted as standard protocol.

2.3.4 Antigen Detection

Sections were washed three times in TBS then placed into humidified slide boxes. Non-specific protein interactions were blocked and secondary antibody host interactions neutralised, by application of 200µL per section 1% w/v Bovine Serum Albumin (BSA; Sigma) in 75% v/v TBS, 25% v/v normal serum (serum of secondary antibody host) and incubation for 90 minutes on a slow moving flatbed shaker. Blocking solution was tapped off slides prior to 200µL application of either primary antibody or isotype control at optimal concentrations pre-determined by titration (described in section 2.3.4.1). Antibody dilutions were performed with 1% w/v BSA in TBS and sections were incubated overnight at 4˚C on a slow moving flatbed shaker, to probe for antigens.

2.3.4.1 Titration of Primary Antibodies

Prior to first experimental use, all primary antibodies were titrated to determine optimal experimental concentration. Table 2-3 gives titration ranges investigated for each antibody alongside optimal experimental concentrations and antibody details. Primary antibody titration is necessary to address the issue of sensitivity (false negativity) of IHC staining protocols (True, 2008). During method optimisation the issue of specificity (false positivity) was also addressed by assessment of isotype and negative controls and secondary antibody binding as detailed in section 2.3.4.2.

2.3.4.2 Isotype and Negative Controls, and Secondary Antibodies

Isotype control sections were run alongside primary antibody sections at matched protein concentrations. Primary antibody binding was only seen to be specific when no binding was observed on matched isotype control sections.

Primary antibody solution was replaced by 1% BSA in TBS on negative control sections. An absence of IHC staining on these sections confirmed that secondary antibody binding was specific to the presence of bound host species proteins (primary antibody or isotype control) (True, 2008).

Both primary and secondary antibody solutions were replaced by 1% BSA in TBS on double-negative control sections. An absence of IHC staining on these sections confirmed that positivity was not as a result of endogenous peroxidase or endogenous biotin activity (True, 2008). Double-negative sections also provided reference for background stain levels as a result of non-specific absorption of conjugates to tissue sections. Details of isotype controls and secondary antibodies used are given in Table 2-3.

Primary Antibody				Biotinylated Secondary Antibody		Control
Target Antigen	Clonality (Isotype)	Titration Range	Optimal Experimental Dilution (Concentration)	Clonality	Dilution (Concentration)	Isotype
IL-1β	M-M (IgG2b)	1:50 - 1:8000	1:50 (40.00µg/mL)	Rabbit anti-Mouse	1:400 (5µg/mL)	IgG2b
IL-16	R-P	1:50 - 1:2000	1:750 (0.67µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CCL2	R-P	1:50 - 1:2000	1:500 (1.00µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CCL3	R-P	1:50 - 1:8000	1:4000 (0.05µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CCL4	R-P	1:500 - 1:16000	1:2000 (0.25µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CCL7	R-P	1:500 - 1:16000	1:10000 (0.10µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CXCL8	R-P	1:50 - 1:1000	1:100 (1.00µg/mL)	Goat anti-Rabbit	1:500 (2µg/mL)	R-P
CD4	M-M (IgG1)	1:250 - 1:4000	1:500 (2.00µg/mL)	Rabbit anti-Mouse	1:400 (5µg/mL)	IgG1
CCR1	M-M (IgG2b)	1:250 - 1:4000	1:1000 (0.50µg/mL)	Rabbit anti-Mouse	1:400/5µg/mL	IgG2b
CXCR1	M-M (IgG1)	1:250 - 1:4000	1:2000 (0.50µg/mL)	Rabbit anti-Mouse	1:400 (5µg/mL)	IgG1
CXCR2	M-M (IgG1)	1:250 - 1:4000	1:500 (2.00µg/mL)	Rabbit anti-Mouse	1:400 (5µg/mL)	IgG1

Table 2-3 Primary Antibodies, Secondary Antibodies and Isotype Controls Used in Immunohistochemistry

M-M, mouse monoclonal; R-P, rabbit-polyclonal; IgG, immunoglobulin. Prior to experimental use all primary antibodies were titrated to determine optimal experimental concentrations. Immunohistochemistry sections were run alongside matched isotype control sections and secondary antibodies were used at supplier recommended concentrations. All Immunohistochemistry antibodies and isotype controls were supplied by Abcam.

2.3.5 Detection of Bound Primary Antibody

Sections incubated with primary antibody and isotype control were washed separately to prevent any cross reaction, three times in TBS. Detection of bound primary antibody or isotype control was by application of 200µL per section of relevant biotinylated secondary antibody (Table 2-3). Antibody dilutions were performed with 1% w/v BSA in TBS and sections incubated for 30 minutes on a slow moving flatbed shaker.

2.3.6 Visualisation of Bound Secondary Antibody

Sections were washed three times in TBS. 2 – 3 drops of A.B.C. Elite Reagent (Vector Laboratories) was applied per section and incubated for 30 minutes on a slow moving flatbed shaker. Following three washes in TBS, 200µL of 0.08% v/v hydrogen peroxide in 0.65mg/mL 3-3'-diaminobenzidine tetrahydrochloride (DAB) in TBS was applied per section and incubated for 20 minutes on a flatbed shaker. Sections were washed twice in deionised water for 1 minute then counter-stained by immersion in Mayer's Haematoxylin (Leica) for 60 seconds and blued under running tap water for 5 minutes.

2.3.7 Mounting of Sections

Sections were dehydrated, cleared and mounted as described previously in section 2.2.2.

2.3.8 Microscopy and Image Capture

Tissue sections were visualised using an Olympus BX60 research microscope. Images were captured using QCapture Pro v8.0 software (MediaCybernetics).

2.3.9 Evaluation of Immunohistochemistry

Evaluation of immuno-detection of target antigens was by counting immuno-positive cells. Within the NP 200 cells were counted and the number of immuno-positive cells expressed as a percentage of this. Cells were counted using x200 magnification as shown in Figure 2-3.

2.4 Cell culture

2.4.1 Cell Culture Techniques

The culture of mammalian cells *in vitro* is a fundamental technique that facilitates the investigation of cell behaviour under defined conditions. Modification of these conditions, for example by addition of a specific cytokine or down-regulation of a specific gene, allows the effect of that variable to be examined and compared to normal physiological (basal) cell behaviour.

2.4.1.1 Primary Nucleus Pulposus Cultures

In these investigations primary NP cultures were utilised in an effort to gain the most physiologically relevant data. Cultures were used for experimental purposes

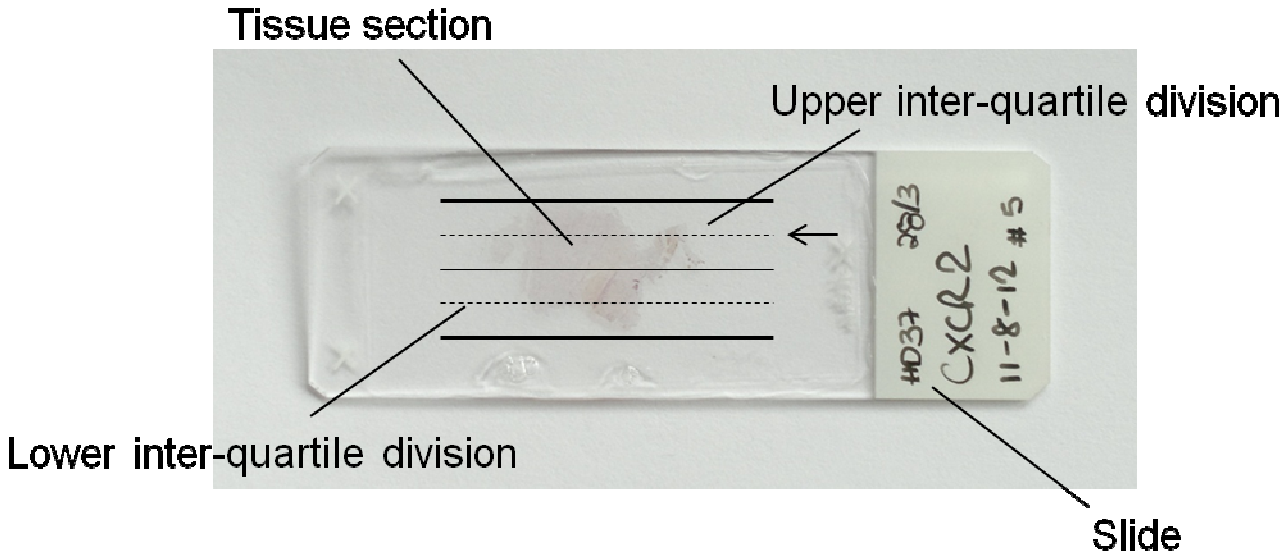


Figure 2-3 Method of Quantification of Immunohistochemistry Detection of Target Cytokines and Chemokines

Cells were counted along the upper inter-quartile division of tissue sections from right to left followed by the lower inter-quartile division from left to right until 200 NP cells had been counted, all cells in the field of vision when 200 was achieved were also included. Cells along the periphery of tissue sections were excluded from the cell count as were cells in any tissue section folds or creases. Any infiltrating cells present in tissue sections were excluded from the cell count.

up to and including passage 2, however even under short-term primary culture NP cells dedifferentiate towards a fibroblastic phenotype. Three-dimensional (3D) culture systems provide a means of maintaining the morphological and biochemical properties of primary NP cultures and even for restoring these features following dedifferentiation during monolayer expansion. Here, the encapsulation of NP cells in alginate beads was investigated alongside monolayer culture, in an effort to develop a suitable *in vitro* model in which to investigate NP cell behaviour.

2.4.1.2 Alginate Bead Culture

Alginate bead culture is a 3D system originally developed as a model for studying chondrocyte behaviour (Hauselmann *et al.*, 1992). The system is based on the material properties of alginic acid from brown seaweed, which forms a viscous liquid in solution and can be polymerised in the presence of divalent cations. These material properties facilitate the incorporation of isolated cells prior to polymerisation, and the semi-solid support formed post polymerisation promotes the correct 3D orientation of cells. This enhances maintenance of the parent phenotype. Further, alginate beads can be easily dissolved by addition of chelating agents providing a means of recovering encapsulated live cells for analysis.

The de-differentiation of articular chondrocytes in monolayer culture and their subsequent re-differentiation following encapsulation and culture within alginate beads has been well characterised (Bonaventure *et al.*, 1994; Lemare *et al.*, 1998). Alterations in the expression of phenotype markers are observed as early as passage 1 in monolayer cultures. Decreased expression of type II collagen and a concordant increase in type I indicates a shift towards a fibroblastic phenotype (Bonaventure *et al.*, 1994; Lemare *et al.*, 1998). Following expansion in monolayer to passage 2, cultures seeded to alginate beads show a further phenotype shift. Increased type II collagen expression and a concordant decrease in type I indicates re-differentiation back towards the chondrocyte phenotype (Bonaventure *et al.*, 1994; Lemare *et al.*, 1998). Morphological and biochemical characteristics 14 days post alginate encapsulation closely resemble those of the parent phenotype (Hauselmann *et al.*, 1992; Bonaventure *et al.*, 1994; Lemare *et al.*, 1998). The development of this culture system has meant that expansion in monolayer prior to re-differentiation in alginate can be used to generate sufficient cell numbers to permit extensive *in vitro* chondrocyte studies.

NP cells are often referred to as 'chondrocyte-like' since morphological and physiological similarities exist. As such the alginate bead culture system has been successfully applied to NP primary culture and similar phenotypic maintenance and

re-differentiation patterns to those seen in chondrocytes are observed (Baer *et al.*, 2001; Wang *et al.*, 2001; Le Maitre *et al.*, 2005).

2.4.2 Nucleus Pulposus Cell Culture

Isolated cells were seeded to 75cm^2 filter-cap cell culture flasks (Nunc) containing Complete Media (SFM plus 10% v/v Heat Inactivated Foetal Bovine Serum (Gibco)). Following passage 1, supplementation of complete media with penicillin, streptomycin and amphotericin B was decreased to 100U/mL, 100ug/mL and 250ng/mL respectively. Cultures were maintained at all times at 37°C and 5% CO_2 in a humidified environment. Cell culture media were changed three times weekly unless specified otherwise.

2.4.2.1 Sub-Culture

Monolayer cultures were maintained up to 80% confluence before passaging: Culture media was removed and 1x Phosphate Buffered Saline (PBS; Gibco) was used to wash the cells removing all traces of serum. Cultures were incubated with 3mL 0.05% Trypsin-EDTA (Gibco) per 75cm^2 flask at 37°C until detachment of cells was achieved. The Trypsin-EDTA was inactivated by the addition of an equal volume of Complete Media. The resulting cell suspension was seeded at a 1:3 split ratio. Following expansion in monolayer to P2, NP cells were subjected to 48 hour cytokine/chemokine treatment in monolayer and/or seeded to alginate culture as described in sections 2.4.3 and 2.4.5.

2.4.3 IL-1β, CCL2 and CXCL8 Treatment of Monolayer Cultures

NP cells were cultured in monolayer up to 80% confluence at passage 2. Following trypsinisation as described in section 2.4.2.1 and centrifugation at 400g for 10 minutes, the cell pellet was re-suspended in Complete Media and cells counted as described in section 2.1.5. Cells were seeded to 6 well plates at a density of 1 x 10^5 cells/well. The 6 well plates were incubated overnight at 37°C to allow adherence of cells. Complete media was removed and replaced with 1mL per well complete media supplemented with 0, 1, 10 or 100 ng/mL of treatment. Each treatment was performed in triplicate and cultures were incubated for 48 hours prior to total RNA extraction. Recombinant human cytokines and chemokines were supplied by Peprotech.

2.4.4 Total RNA Extraction from Monolayer Cultures

Complete media was removed and the wells washed in PBS. 1mL trizol was added per well and the plates incubated at room temperature for 5 minutes. The trizol cell suspension was transferred to 1.5mL tubes and total RNA was isolated as previously described in section 2.1.5, up to centrifugation to recover RNA precipitate following isopropanol incubation at -80°C. At this stage monolayer

extractions were not subjected to DNase treatment and Qiagen Min-Elute Clean-up, this was replaced by washing with ethanol; RNA pellets were re-suspended in 75% ethanol (Sigma) and re-centrifuged at 7500g for 15 minutes at 4°C. Supernatant was removed and remaining RNA pellet air dried on ice for approximately 20 minutes. RNA pellets were then re-suspended in 14µL sterile deionised water and used immediately for cDNA synthesis as described in section 2.5.2.

2.4.5 Seeding Nucleus Pulposus Cells to Alginate Culture

Monolayer cultures were trypsinised as described in section 2.4.2.1 and centrifuged at 400g for 10 minutes, following which cells were re-suspended in complete media and counted as described in section 2.1.5. Cells were re-centrifuged at 400g for 10 minutes, supernatant was discarded and the remaining pellet was re-suspended in sterile 1.2% w/v low viscosity alginic acid (Sigma) in 0.15M sodium chloride (Fisher Scientific) at a density of 2.0×10^6 cells/mL. Alginate beads were formed by 'dropping' the alginate cell suspension through a 19 gauge needle into 12-well plates containing 2.5mL sterile 200mM calcium chloride (Fisher Scientific) per well. Plates were incubated at 37°C for 15 minutes to allow polymerisation of beads then each well was washed twice in 2.5mL of 0.15M sodium chloride once in 2.5mL of complete media. Alginate bead cultures were maintained for 14 days in complete media prior to cytokine/chemokine treatment. Cell culture media was changed twice weekly.

2.4.6 IL-1β, IL-16, CCL3 and CCL7 Treatment of Alginate Cultures

2 Alginate beads were placed into 96-well plates and 200µL complete media supplemented with 0, 1, 10 or 100ng/mL of treatment was added per well. IL-1β treatments were performed at additional treatment concentrations of 1, 10 and 100 pg/mL. Each treatment was performed in triplicate and the cultures were incubated for 48 hours prior to total RNA extraction.

2.4.7 Total RNA Extraction from Alginate Cultures

Culture supernatant from alginate beads subjected to cytokine/chemokine treatment was removed into 1.5mL sterile tubes and frozen at -80°C for use in further Luminex studies as described in section 2.6. Total RNA Extraction from Alginate Cultures was by Trizol or RNeasy method:

2.4.7.1 Optimisation of Trizol RNA Extraction Protocol

The downstream applications of cDNA synthesis and real-time polymerase chain reaction (PCR) are techniques based on enzyme activity. As such they are sensitive to reaction conditions and RNA samples generated must be of sufficient quantity and quality to support these applications.

In simple extractions from monolayer or suspension cultures, care must be taken to minimise carry-over of the commonly used phenolic reagent, trizol, as phenol contamination can inhibit PCR. Extractions from alginate bead cultures are further complicated by the additional procedures required to recover cells and subsequent exposure to potential contamination from EDTA contained within the alginate dissolving buffer. In initial investigations, contaminants in RNA samples extracted from alginate cultures were seen to severely inhibit PCR.

The effect of culture size and cell density on carry-over contamination was assessed: Trizol RNA extractions were performed on 1, 2, 4, 6, 8, 10 and 12 alginate beads seeded at 1, 2 and 4 x 10^6 cells/mL, with and without RNA purification by Qiagen Min-Elute Clean-Up Kit. Real-time PCR for GAPDH was performed on cDNA generated from all RNA samples. Amplification was not observed in any PCR reaction without prior RNA purification. Purified samples from 1, 2 and 4 beads generated amplification plots however, increasing bead number delayed amplification (Figure 2-4), indicating increased inhibition. The earliest C_T values were seen in samples from 1 bead seeded at 2 x 10^6 cells/mL.

RNA samples extracted from 1 bead were subjected to nanodrop spectrophotometer analysis. Absorbance was measured at wavelengths of 260 and 280 nm. The ratio of these absorbances can be used as a measure of RNA purity, and samples seeded at 2 x 10^6 cells/mL were seen to have greater purity than those seeded at 1 or 4 x 10^6 cells/mL. This likely explains the lower C_T values generated by these samples. RNA integrity was also confirmed by 1% agarose gel electrophoresis and ethidium bromide staining in these samples (not shown). 1 bead seeded from 2 x 10^6 cells/mL alginate suspension was selected as optimal for experimental use.

2.4.7.2 Optimisation of RNeasy RNA Extraction Protocol

1mL of 2 x 10^6 cells/mL alginate suspension was seen to generate 50 polymerised beads, equating to RNA from 0.4 x 10^5 cells per extraction. Certain genes were difficult to detect in these samples, possibly due to low copy number. A further RNA extraction protocol was developed; using Qiagen RNeasy Mini Kit – instead of trizol, to prevent phenol contamination, and a shortened incubation in alginate dissolving buffer during cell recovery - to limit EDTA contamination. By this method, it was possible to extract RNA from 2 beads (0.8 x 10^5 cells) and low copy number genes were detectable in these samples.

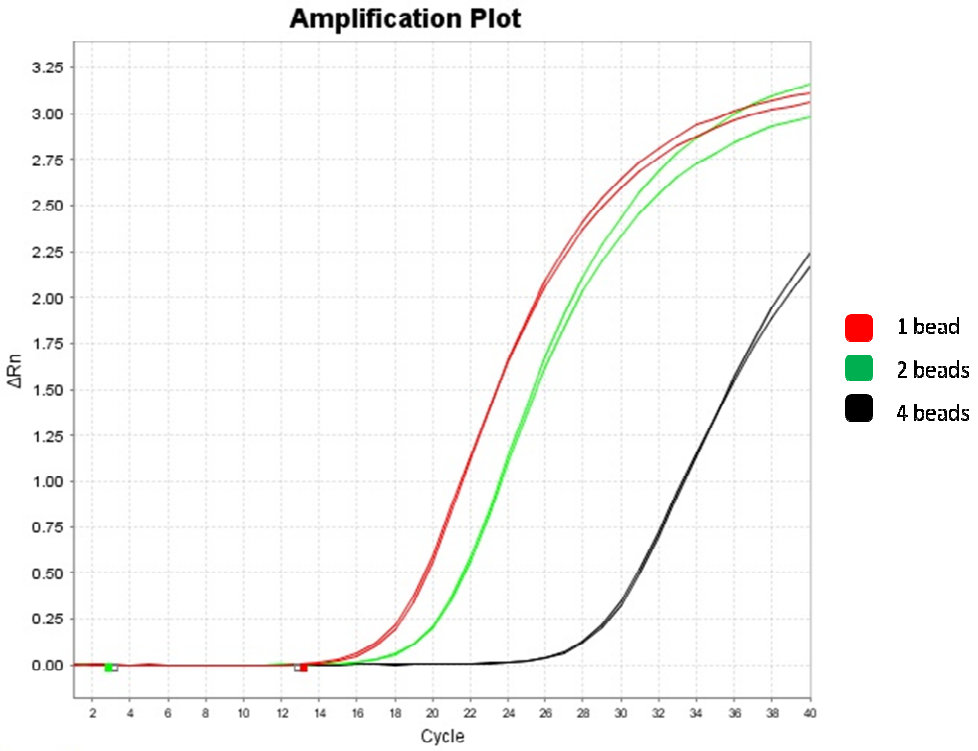


Figure 2-4 Amplification Plots Generated using cDNA Samples Reverse Transcribed from RNA Extracted by Trizol Method from Alginate Cultures

Real-time PCR for GAPDH on cDNA generated from RNA extracted by Trizol method (incorporating Qiagen Min-Elute Clean-up) generated amplification plots in samples derived from 1, 2 and 4 alginate beads. No amplification was seen in cDNA samples derived from RNA extracted from greater than 4 beads.

2.4.7.3 Trizol RNA Extraction Standard Protocol

1 alginate bead was removed from each well of the 96-well plates into RNase free 1.5mL tubes. 500µL of alginate dissolving buffer (55mM sodium citrate (Sigma), 30mM EDTA (Fisher Scientific), 0.15M NaCl, pH 6) was added per tube and samples incubated, rotating at 37°C for 20 minutes to dissolve alginate. Samples were centrifuged at 600g for 15 minutes at 4°C to recover cells. Supernatant was removed and the remaining pellet re-suspended in 500µL 0.06% w/v type I collagenase (Sigma) in SFM, samples were incubated rotating at 37°C for 20 minutes to digest ECM. Samples were centrifuged at 600g for 15 minutes at 4°C. Supernatant was removed and the remaining pellet was re-suspended in 1mL Trizol and incubated at room temperature for 5 minutes. Total RNA was isolated as described previously in section 2.1.5 however, RNA extractions from alginate cultures were not subjected to DNase treatment therefore recovered RNA pellets were re-suspended in 100µL not 87.5µL, sterile deionised water and used immediately in Qiagen Min-Elute Clean-up to purify and recover RNA samples. RNA was eluted in 14µL sterile deionised water and used immediately for cDNA synthesis as described in section 2.5.2.

2.4.7.4 RNeasy RNA Extraction Standard Protocol

2 Alginate beads were removed from each well of the 96-well plates into RNase free 1.5mL tubes. 500µL of alginate dissolving buffer was added per tube and samples incubated rotating at 37°C for 10 minutes to dissolve alginate. Samples were centrifuged at 600g for 10 minutes at 4°C to recover cells. Supernatant was discarded and the remaining pellet was re-suspended in 500µL 0.06% w/v type I collagenase (Sigma) in SFM, and incubated rotating at 37°C for 10 minutes to digest ECM. Samples were centrifuged at 600g for 10 minutes at 4°C. Supernatant was discarded and the remaining cell pellet was used immediately for RNA extraction by Qiagen RNeasy Mini Kit as per manufacturer's protocol. RNA samples were eluted in 30µL RNase free water and used immediately in cDNA synthesis as described in section 2.5.2.

2.5 Quantitative Polymerase Chain Reaction

2.5.1 The Principle of qPCR

Quantitative polymerase chain reaction (qPCR) is widely used to detect and quantify gene expression. It involves reverse transcription (RT) of mRNA into cDNA followed by PCR to amplify a specific target cDNA molecule, which is quantified in real-time by the accumulation of fluorescence after each amplification cycle (Orlando *et al.*, 1998).

Two distinct types of detection chemistry are used in qPCR, namely probe or non-probe. Probe based detection is amplicon specific, it utilises fluorescent probes that only generate a signal following replication of their complementary target, as this amplification removes an associated quencher molecule (Figure 2-5). Non-probe detection methods rely on fluorescent intercalating agents that emit minimal fluorescence in reaction solution but increase emission following intercalation with synthesised double stranded DNA as polymerisation proceeds. Verification of PCR products is required in non-probe detection methods and so a disassociation curve of the amplicon is generated following the final PCR cycle. Fluorescence is plotted as a function of temperature, identical amplicons are seen to dissociate at the same temperature (Orlando *et al.*, 1998), thus confirming that only primer specific amplification has occurred.

There are two established methods of PCR product quantification, relative and absolute. Relative quantification is used to determine mRNA quantity relative to that of internal reference genes in separate samples; or changes in mRNA expression across multiple samples under varying experimental conditions, relative to both internal reference gene expression and basal expression of the target gene. Several mathematical algorithms have been developed to produce the corrected relative expression ratio; these include relative quantification without primer amplification efficiency correction – the comparative quantification method ($2^{-\Delta CT}$ /$2^{-\Delta\Delta CT}$ method) (Livak & Schmittgen, 2001), and relative quantification with primer amplification efficiency correction – the Pffafl method (Pfaffl, 2001).

In order for the former model to be valid, the primer amplification efficiencies of the target and internal reference genes must be approximately equal (within 10%), which is not always the case. Differences in primer amplification efficiencies are taken into consideration in the latter model. It is generally considered that relative quantification is adequate for investigating physiological changes in gene expression levels, though care must be taken to select stably expressed internal reference genes that are not affected by any experimental variation under investigation (Dheda *et al.*, 2004; Radonic *et al.*, 2004).

Absolute quantification can also be used to determine mRNA quantity in separate samples for comparative analysis, or changes in target mRNA quantity under varying experimental conditions. Absolute quantification uses a standard curve of C_T values, generated from a dilution series of an external standard with known initial target copy number. Data generated is in the form of absolute starting quantity of target gene mRNA within an unknown sample, however reliability in this

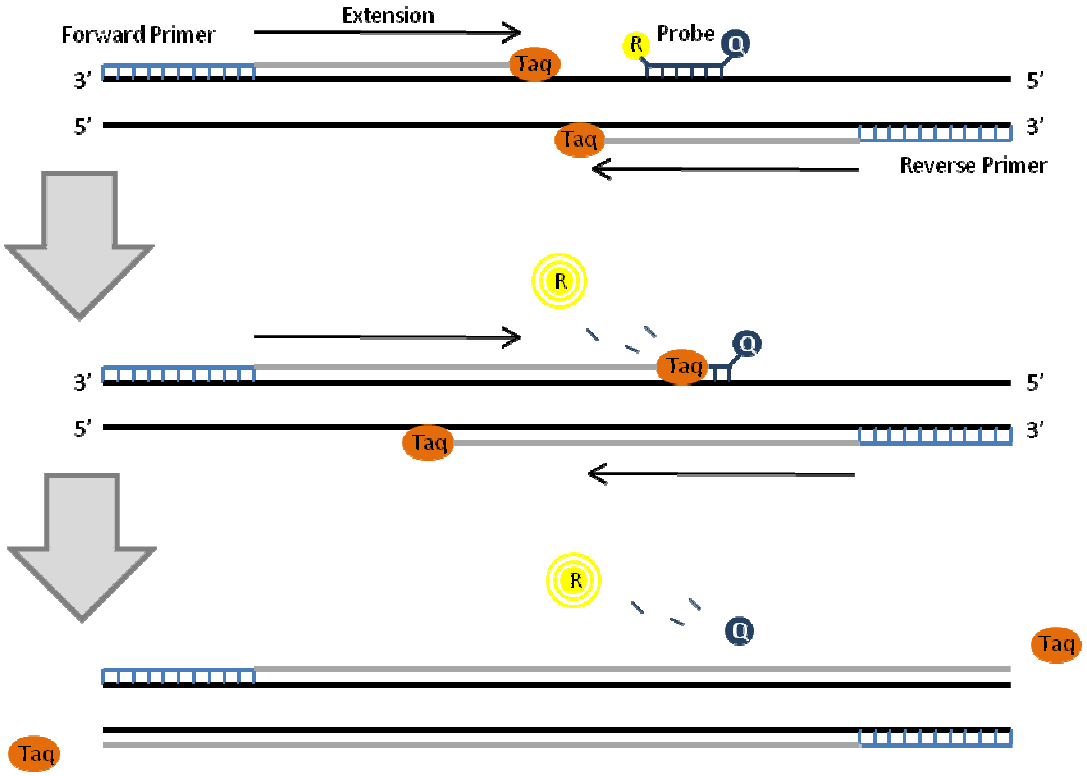


Figure 2-5 Schematic Representation of cDNA Amplification and Generation of Fluorescence by Taqman Real-Time Polymerase Chain Reaction

Taqman detection monitors the degradation of a sequence specific dual labelled probe following each cycle of polymerase chain reaction. In the initial phase of each thermo-cycle double stranded cDNA is denatured at 95°C, subsequent cooling to 60°C facilitates annealing of primers and probes to complimentary base specific sequences. Taqman assays are designed such that probe binding is up-stream of primer binding and within the target amplicon. Incubation at 60°C permits *Taq* polymerase activity and replication of the target DNA molecule proceeds. At the point where extension meets probe binding, 5'-exonuclease activity of the *Taq* enzyme sequentially removes probe bases. This cleavage of the probe separates the fluorescent reporter dye from the quencher, increasing fluorescence emission within the reaction. Sample emission levels are measured at completion of each thermo-cycle and an amplification plot is generated.

method relies on the accuracy of standards used (Whelan *et al.*, 2003; Larionov *et al.*, 2005).

2.5.2 cDNA Synthesis

RNA samples were converted to cDNA using Moloney Murine Leukaemia Virus reverse transcriptase (Bioline): RNA samples were incubated at 60°C for 5 minutes to denature RNA. RT mastermix was added immediately, 36µL per 14µL RNA sample or 20µL per 30µL RNA sample, made up as shown in Table 2-4. Samples were incubated at 42°C for 1 hour to permit cDNA synthesis, then 10 minutes at 80°C to inactivate RT enzyme. cDNA samples were diluted 1:10 by addition of 450µL sterile deionised water and stored at -20°C for use in qPCR.

For 1 Reaction:	14µL RNA Sample (µL)	30µL RNA Sample (µL)
Deoxynucleotide triphosphates (dNTPs) 40mM (Bioline)	1.5	1.5
Random Hexamers (AB)	1.0	1.0
Bioscript 5x RT Buffer (Bioline)	5.0	5.0
Bioscript RT Enzyme (Bioline)	0.5	0.5
Sterile distilled H_2O	28.0	12.0
	36.0	20.0

Table 2-4 Reverse Transcription Mastermix used in cDNA Synthesis Reactions

2.5.3 Taqman cDNA Low Density Array

Prior systematic review of literature identified 25 cytokines and 18 chemokines that are implicated in the pathology of connective tissue degeneration (Chapter 1, section 01.4, summarised in Table 1-4). These targets were selected for inclusion, and some related family members that have not yet been investigated in connective tissues were also included, alongside associated receptors, accessory proteins and activating enzymes for each cytokine or chemokine, on a Taqman cDNA LDA that was custom manufactured on FAST 96-well plate setup, by Applied Biosystems. Five stably expressed internal reference genes, from a previous study into expression of commonly used reference genes in human NP cells (Le Maitre, 2003), were also selected for LDA inclusion. LDA design is shown in Figure 2-6.

Specific, probe based Taqman qPCR detection was used on cDNA samples from DE NP cells. Separate reaction mastermixes were prepared for each cDNA

	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	ACTB	PGK	IL-17RA	IL-22RαI	IFN-γRα	gp130	CXCL10	CCL19	CCR3
B	IL-1α	IL-15	IL-17F	CSF3	IL-6R	IL-17RB	IL-23R	IFN-γRβ	CXCL1	CCL2	CCL20	CCR5
C	IL-1β	IL-16	IL-18	IFN-γ	IL-7R	IL-17RC	IL-12Rβ1	OSMR	CXCL2	CCL3	CX3CL1	CCR6
D	IL-4	IL-17A	IL-20	OSM	IL-10Rα	IL-17RD	TNF-R55	LIFRα	CXCL3	CCL4	CXCR1	CCR7
E	IL-6	IL-17B	IL-23	LIF	IL-10Rβ	IL-18Rα	TNF-R75	IL-1Ra	CXCL5	CCL5	CXCR2	CCR8
F	IL-7	IL-17C	TNF-α	IL-1RI	IL-11Rα	IL-18Rβ	GM-CSFRα	IL-1RAcP	CXCL6	CCL7	CXCR3	CX3CR1
G	IL-10	IL-17D	TNF-β	IL-1RII	IL-15Rα	IL-20RI	GM-CSFRβ	ICE	CXCL8	CCL8	CCR1	Duffy
H	IL-11	IL-17E	CSF2	IL-4R	CD4	IL-20RII	G-CSFR	TACE	CXCL9	CCL13	CCR2	D6

Figure 2-6 Low Density Array 96-well Plate Setup

Internal reference genes; 18S, GAPDH, HPRT1, ACTB and PGK were included in LDA design alongside 30 cytokine, 18 chemokine, receptors, activating enzymes and signalling accessory proteins. Chemokine systematic nomenclature is used – alternative names are given in Appendix 5.

sample investigated, as shown in Table 2-5, and 20µL per well loaded into LDA plates. Each cDNA sample was run in duplicate. Plates were sealed with adhesive film and kept at 4°C until being run on an Applied Biosystems, StepOnePlus Real-Time PCR machine on FAST programme incorporating 50 cycles of denaturation at 95°C for 1 second followed by annealing and extension at 60°C for 20 seconds.

For 1 Reaction:	(µL)
cDNA	1.0
Taqman FAST Universal Mastermix (Applied Biosystems)	10.0
Sterile deionised water	9.0
	20.0

Table 2-5 qPCR Reaction Mastermix used in Low Density Array Analysis

2.5.4 Analysis of Low Density Array Data

Quantification of gene expression was by comparative quantification method ($2^{-\Delta CT}$). This method was applicable since all primer sequences were pre-validated by manufactures to confirm equality of amplification efficiencies. In order to generate reliable comparable data, particular attention was given to baseline and threshold settings (Figure 2-7) and to the selection of internal reference genes (see section 2.5.4.1). Threshold settings for all 96 LDA targets are given in Appendix 4.

Briefly, analysis was performed by: calculation of the arithmetical mean C_T values of internal reference and the target gene sample duplicates, and calculation of the arithmetical mean of the selected internal reference genes to generate the 'mean reference' value for each sample. ΔC_T values were then calculated by the equation:

$$\Delta C_T = C_T\,(target) - C_T\,(mean\ reference)$$

Target gene expression, relative to internal reference gene expression, was then calculated using the equation:

$$Relative\ gene\ expression = 2^{-\Delta CT}$$

Relative gene expression values were multiplied by 10^6 to give values on an acceptable scale when represented graphically.

2.5.4.1 Internal Reference Gene Selection

Selection of stably expressed internal reference genes is a pre-requisite for the accurate normalisation of real-time qPCR data (Vandesompele *et al.*, 2002). The geNorm applet for Microsoft Excel can be used to determine internal reference

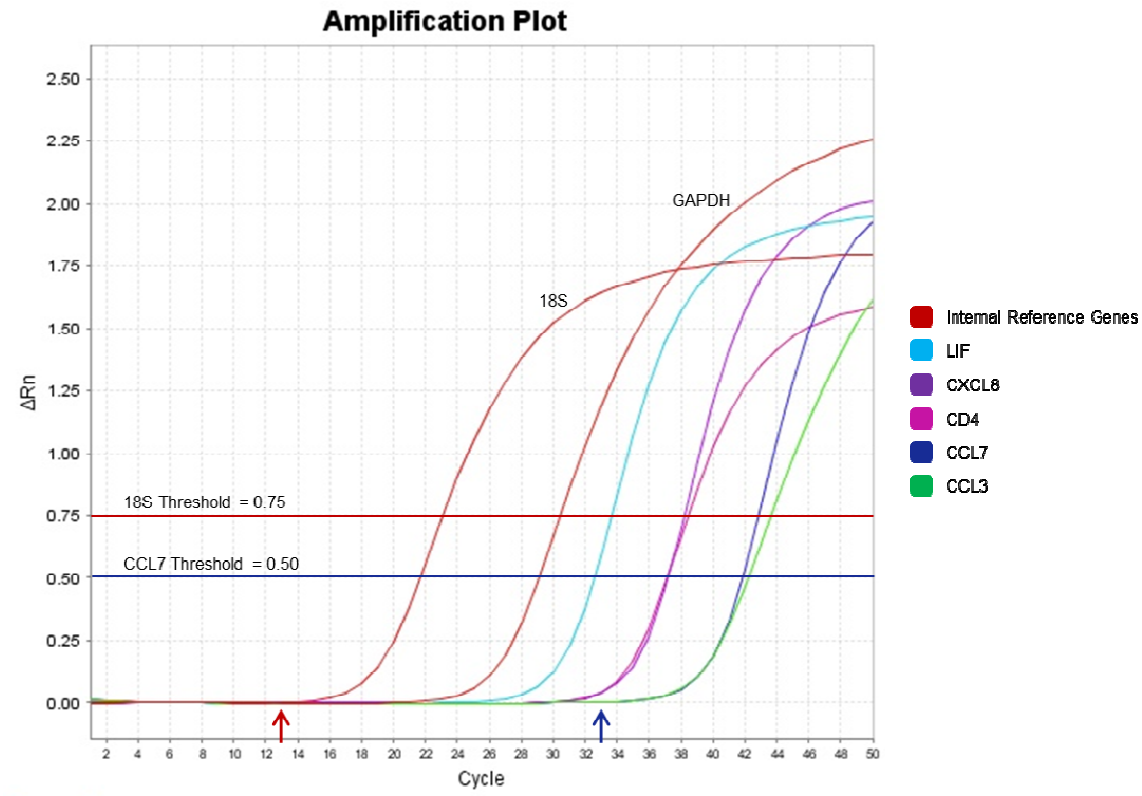


Figure 2-7 Low Density Array Amplification Plot Annotated to Illustrate the Methods used to Set Baselines and Thresholds in qPCR Analysis

Data generated in real-time PCR is expressed as an amplification plot where measured fluorescence emission is plotted as a function of time shown as thermo-cycles completed. Following amplification, and prior to data collection, baselines were set for each target investigated at two cycles prior to visible amplification thus ensuring all assays were normalised for the presence of inherent reaction solution background emission. Thresholds were set within the exponential phase of amplification and maintained across investigations. This factor in particular provides method advantage over standard PCR since data collection in the exponential phase (prior to reaction saturation) makes this method fully quantifiable.

Shown here are the amplification plots of 5 target and 2 internal reference genes following LDA analysis of cDNA generated from DE NP cells. The red arrow indicates baseline setting of 13 for 18S gene analysis - 2 cycles prior to visible amplification at cycle 15, and a threshold setting of 0.75 - within the exponential phase of the plot. The dark blue arrow indicates baseline setting of 33, and threshold is shown at 0.50 for CCL7 gene analysis.

gene stability in a given cDNA sample panel. The geNorm applet calculates the gene expression stability measure (*M*) for each reference gene as the average pair wise variation (*V*) for that gene with all other tested reference genes. This measure relies on the principle that the expression ratio of ideal internal reference genes is identical in all samples, regardless of experimental conditions (Vandesompele *et al.*, 2002; Huggett *et al.*, 2005).

In an ideal experimental system the gene expression stability measure (*M*) should be less than 1.5 (Vandesompele *et al.*, 2002). Use of the geNorm applet allows ranking of test reference genes according to their stability. Exclusion of the least stable, and subsequent re-calculation can be performed stepwise to reach an *M* value of <1.5 in the experimental system investigated.

This analysis was performed on internal reference gene expression data, generated in the 12 real-time qPCR LDA experiments. Briefly, C_T values were measured for each gene at the same threshold in all samples. The raw C_T values were transformed by first subtracting the highest C_T value from all others – generating the 'change in' ΔC_T value, and then applying the equation$2^{-\Delta C_T}$. Transformed data was subjected to geNorm analysis. The gene expression stability measure of the 5 combined internal reference genes included in the LDA was 1.08, and so LDA data was normalised to all 5 internal reference genes.

2.5.5 qPCR on cDNA from Directly Extracted Nucleus Pulposus Cells

Specific probe based Taqman qPCR detection was used on cDNA samples from DE NP cells. Two internal reference genes were selected for use in all experiments to normalise for variations in total RNA in each sample (see section 2.5.7.1).

2µL sample cDNA was loaded in duplicate into 96-well FAST PCR plates (Applied Biosystems). 8µL per well Real-Time PCR mastermix, prepared as shown in Table 2-6, was added. Plates were sealed with adhesive film and kept at 4°C until being run on a StepOnePlus Real-Time PCR machine as described previously in section 2.5.3.

For 1 Reaction:	µL
Taqman Gene Expression Assay	0.5
2x Taqman FAST Mastermix	5.0
Sterile distilled H_2O	2.5
	8.0

Table 2-6 qPCR Reaction Mastermix used in Gene Expression Analysis

2.5.6 Analysis of qPCR data

Real-time PCR data was analysed as previously described in section 2.5.4. Thresholds used in original LDA analysis were maintained across all samples investigated.

2.5.6.1 Internal Reference Gene Selection

The geNorm applet can also be used to determine the minimum number of reference genes required to calculate a reliable normalisation factor. The use of a single reference gene is discouraged since no simple means to confirm that the gene is unaffected by experimental variation exists (Vandesompele *et al.*, 2002). Reference gene instability can be detected in multiple reference gene experimental systems, as alteration in expression of one reference gene would affect its expression ratio compared to the others used. For this reason it's important to select multiple internal reference genes (Vandesompele *et al.*, 2002) and to ensure that genes are selected from different functional classes (Radonic *et al.*, 2004). This minimises the chances that the genes will be co-regulated and so equally affected by any experimental variation.

Prior geNorm analysis (section 2.5.4.1), indicated that whilst the 5 internal reference genes analysed by LDA provided an accurate normalisation factor (*M* = 1.08), HPRT1 and ACTB were the least stable and 18S, GAPDH and PGK the most. The pair wise variation (*V*) calculated in geNorm is a measure of the increased accuracy in normalisation factor generation that can be gained from including the next most stably expressed reference gene, according to *M* value rank. The accepted *V* cut-off value is 0.15, below which the inclusion of an additional reference gene will have no significant effect but above which inclusion is required for accurate normalisation (Vandesompele *et al.*, 2002). Pair wise variation in the 3 most stably expressed genes (*V*2/3) was 0.132 indicating little increase in normalisation accuracy could be gained from using 3 internal reference genes rather than 2. Therefore, eukaryotic 18S rRNA (18S) and glyceraldehyde-3-phosphate dehydrogensase (GAPDH) were selected as internal reference genes for qPCR data analysis, based on excellent gene expression stability (*M* = 0.402) and no requirement for an additional third reference gene (*V* = 0.132).

2.5.7 qPCR on cDNA from Monolayer and Alginate Cultures

Specific probe based Taqman qPCR detection was used alongside relative quantification by comparative quantification method ($2^{-\Delta\Delta CT}$), as described previously (section 2.5.1). Taqman gene expression assays were selected or designed so as to span exon-exon boundaries ensuring amplification of cDNA from spliced mRNA transcripts only (Table 2-7). Amplification efficiencies of internal

Target Gene	Assay ID	Threshold	Efficiency
18S	Hs99999901_s1	0.75	99.6%
GAPDH	Hs99999905_m1	0.75	99.4%
IL-1β	Hs01555413_m1	0.75	94.7%
IL-16	Hs00189606_m1	0.25	95.3%
CCL2	Hs00234140_m1	0.50	96.4%
CCL3	Hs00234142_m1	0.50	97.7%
CCL7	Hs00171147_m1	0.50	96.2%
CXCL8	Hs00174103_m1	0.50	95.1%
Aggrecan	Forward primer: TCGAGGACAGCGAGGCC Reverse primer: TCGAGGTAGCGTGTAGAGA Probe: ATGGAACACGATGCCTTTCACCACGA	0.75	98.8%
Collagen (II)	Forward primer: GGCAATACGAGGTTCACGTACA Reverse primer: CGATAACAGTCTTGCCCCACTT Probe: CCGGTATGTTTCGTGCAGCCATCCT	0.50	98.5%
MMP-3	Hs00233962_m1	0.75	97.6%
MMP-13	Hs00233992_m1	0.40	96.5%
ADAMTS-4	Forward primer: ACTGGTGGTGGCAGATGACA Reverse primer: TCACTGTTAGCAGGTAGCGCTTT Probe: ATGGCCGCATTCCACGGTG	0.50	95.1%

Table 2-7 Details of Taqman Assays used to Analyse Gene Expression in Monolayer and Alginate Cultured Nucleus Pulposus Cells under Basal and Cytokine Stimulated Conditions

Prior to experimental use the amplification efficiency of reactions by each gene expression assay was confirmed under experimental conditions for monolayer and alginate derived samples. All gene expression assays were supplied by Applied Biosystems.

reference gene expression assays and target gene expression assays were confirmed to be equivalent prior to experimental use (section 2.5.8.1). qPCR was performed as described previously in section 2.5.5. Target gene expression was investigated in monolayer and alginate cultures following cytokine or chemokine treatment, for specified target genes as shown in Table 2-8.

2.5.7.1 Reaction Amplification Efficiency

Prior to experimental use, the amplification efficiency of each gene expression assay was confirmed to be within 10% of internal reference gene amplification efficiency by template titration. Amplification efficiencies were confirmed using template cDNA from both monolayer and alginate culture derived samples. Thresholds used for template titration were maintained across all experimental samples investigated (Table 2-7).

Briefly, template cDNA was prepared so that an undiluted (neat) sample was run alongside 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions. Amplification plot data was analysed by plotting the experimental C_T values for each dilution against Log_2 of cDNA concentration (expressed as a percentage of the neat). The amplification efficiency was calculated as the slope of the best linear fit of the expected increase in C_T versus the C_T value obtained. A slope of -1 corresponds to one additional cycle required to reach the threshold after halving template copy number, and would indicate 100% amplification efficiency (Martin ***et al.***, 2001).

2.5.8 Analysis of qPCR Data

Quantification of gene expression was by comparative quantification method ($2^{-\Delta\Delta CT}$). This method was applicable since all primer sequences were pre-validated by template titration to confirm equality of amplification efficiencies. Particular attention was given to baseline and threshold settings (Figure 2-7) and to confirming the stability of internal reference gene expression under treatment conditions (see section 2.5.9.1).

Briefly, ΔC_T values were obtained as described previously (section 2.5.4). The arithmetical mean ΔC_T and standard error of the mean (ΔC_T SE) for each triplicate data set (untreated control and treatment groups) was calculated. $\Delta\Delta C_T$ values were calculated using the equation:

$$\Delta\Delta C_T = mean\ \Delta C_T(treatment\ group) - mean\ \Delta C_T\ (untreated\ control)$$

Target gene expression, relative to internal reference gene and basal expression, was calculated using the equation:

Table 2-8 qPCR Investigation of Target Gene Expression in Monolayer and Alginate Cultured Nucleus Pulposus Cells Subjected to 48 Hour Cytokine or Chemokine Treatment

Cultured nucleus pulposus cells were obtained from surgical intervertebral disc tissue samples. Full details of tissue samples used in these investigations can be found in Appendix 1. Where target gene expression was investigated, but not detected under basal conditions, samples are shown in *italics*. * indicates RNA extraction performed by RNeasy method, all other RNA extractions were performed by trizol method.

Target Gene	Cytokine/Chemokine Treatment						
	Monolayer Cultures			Alginate Cultures			
	IL-1β	CCL2	CXCL8	IL-1β	IL-16	CCL3	CCL7
IL-1β	HD2 HD4 HD6 HD8 HD9	HD2 HD4 HD6	HD2 HD4 HD6	*HD9* *HD23* *HD54*	*HD8* *HD23* *HD56*	*HD8* *HD23* *HD56*	*HD8* *HD23* *HD56*
IL-16	Not Investigated			HD9 *HD23* *HD54* HD79* HD85* HD86* HD89*	HD8 *HD23* *HD56* HD79* HD85* *HD89**	HD8 *HD23* *HD56*	HD8 *HD23* *HD56*

Target Gene	Cytokine/Chemokine Treatment						
	Monolayer Cultures			Alginate Cultures			
	IL-1β	CCL2	CXCL8	IL-1β	IL-16	CCL3	CCL7
CCL2	HD2 HD4 HD6 HD8 HD9	HD2 HD4 HD6	HD2 HD4 HD6	HD9 HD23 HD54	HD8 HD23 HD56	HD8 HD23 HD56	HD8 HD23 HD56
CCL3	Not Investigated			*HD9* *HD23* *HD54*	*HD8* *HD23* *HD56*	*HD8* *HD23* *HD56*	*HD8* *HD23* *HD56*
CCL7	Not Investigated			HD9 HD23 HD54 HD75	HD8 HD23 HD56	HD8 HD23 HD56	HD8 HD23 HD56
CXCL8		HD2 HD4 HD6 HD8 HD9		HD2 HD4 HD6	HD2 HD4 HD6	HD9 HD23 HD54	HD8 HD23 HD56

Table 2-8 Continued from previous page.

Target Gene	Cytokine/Chemokine Treatment						
	Monolayer Cultures			Alginate Cultures			
	IL-1β	CCL2	CXCL8	IL-1β	IL-16	CCL3	CCL7
Aggrecan		HD2 HD4 HD6		HD2 HD4 HD6	HD2 HD4 HD6	HD9 HD23 HD54 HD75 HD79* HD85* HD86* HD89*	HD8 HD23 HD56 HD79* HD85* HD89*
Type II Collagen		Not Investigated		*HD9* *HD23* *HD54* HD79* *HD85** HD86* HD89*	*HD8* *HD23* *HD56* HD79* HD85* HD89*	*HD8* *HD23* *HD56*	*HD8* *HD23* *HD56*
MMP-3		HD2 HD4 HD6		HD2 HD4 HD6	HD2 HD4 HD6	HD9 HD23 HD54	HD8 HD23 HD56

Table 2-8 Continued from previous page.

Target Gene	Cytokine/Chemokine Treatment						
	Monolayer Cultures			Alginate Cultures			
	IL-1β	CCL2	CXCL8	IL-1β	IL-16	CCL3	CCL7
MMP-13		HD2 HD4 HD6		HD2 HD4 HD6	HD2 HD4 HD6	HD9 HD23 HD54	HD8 HD23 HD56
ADAMTS-4		HD2 HD4 HD6		HD2 HD4 HD6	HD2 HD4 HD6	*HD9* *HD23* *HD54* HD79* HD85* HD86* HD89*	*HD8* *HD23* *HD56* HD79* HD85* *HD89**

Table 2-8 Continued from previous page.

$$Relative\ gene\ expression = 2^{-\Delta\Delta CT}$$

Relative gene expression was represented graphically and error bars representing standard error of ΔC_T values for each triplicate data set were calculated using the equations:

$$Positive\ Error\ Bar = 2^{(-\Delta\Delta CT + \Delta CT\ SE)} - 2^{-\Delta\Delta CT}$$

$$Negative\ Error\ Bar = 2^{-\Delta\Delta CT} - 2^{(-\Delta\Delta CT - \Delta CT\ SE)}$$

2.5.8.1 Internal Reference Gene Stability

Internal reference gene stability was confirmed under treatment conditions using the geNorm applet, as described previously (section 2.5.4.1), to calculate the gene expression stability measure (*M*; Table 2-9).

Treatment	Average Stability Expression Measure (*M*)
IL-1β	HD2; *M* = 0.406 HD4; *M* = 1.454 HD6; *M* = 0.531
IL-16	HD8; *M* = 0.685 HD23; *M* = 0.830 HD56; *M* = 1.364
CCL2	HD2; *M* = 1.031 HD4; *M* = 0.682 HD6; *M* = 0.418
CCL3	HD8; *M* = 0.609 HD23; *M* = 0.169 HD56; *M* = 1.416
CCL7	HD8; *M* = 0.675 HD23; *M* = 0.709 HD56; *M* = 1.499
CXCL8	HD2; *M* = 0.214 HD4; *M* = 1.372 HD6; *M* = 0.481

Table 2-9 Confirmation of Internal Reference Gene Stability in Cultured Nucleus Pulposus Cells Subjected to 48 Hour Cytokine or Chemokine Stimulation

The geNorm algorithm was used to confirm internal reference gene (GAPDH and 18S) stability under treatment conditions. In ideal experimental systems, *M* = <1.5, providing an accurate normalisation factor for comparative analysis of qPCR data.

2.6 Luminex Multiplex Bead Immunoassay

2.6.1 The Principle of Luminex Technology

Luminex technology is based on the use of 5.6µm polystyrene microspheres (beads) each internally dyed with a unique combination of red and infrared dyes. The combination of the different intensities of the two dyes allows for the identification of each bead by its unique spectral properties when excited by a laser beam (Carson & Vignali, 1999).

The bead surface can be coated with a number of different targets (e.g. antibodies, antigens, oligonucleotide probes). Coated beads can then be used to interrogate samples for the presence or absence of specific analytes. The Luminex platform uses the principles of flow cytometry to stream beads in single file past a pair of lasers (Carson & Vignali, 1999). A red laser is used to excite and therefore identify the specific bead, and a green laser is used to excite and therefore identify any reporter dyes captured on the bead during the assay. As both the bead identification and reporter dye readings are made on each individual bead, a multiplex system can be developed with up to 100 beads (Carson & Vignali, 1999).

2.6.1.1 Immunoassays

Multiplex bead immunoassays are solid phase protein immunoassays (Vignali, 2000). Beads of defined spectral properties are conjugated to capture antibodies directed against the specific analyte/s of interest. These are used to interrogate analyte standards of known concentration alongside test samples. Later analyte specific biotinylated detector antibodies are added and these bind to the appropriate analyte that is immobilised on the bead surface. Streptavidin conjugated to the fluorescent protein, R-Phycoerythrin, is added and binds to the biotinylated detector antibodies, forming a four member solid phase sandwich (Vignali, 2000). Beads are analysed on the Luminex platform by monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin fluorescence (Figure 2-8). A standard curve is generated based on bead readings from standards of known analyte concentration. From this, the concentration of protein in test samples can be determined.

2.6.2 Multiplex Bead Immunoassays

Commercially available Luminex Multiplex Bead Immunoassays (Invitrogen) were performed on supernatant samples recovered from cytokine/chemokine treated alginate cultures (section 2.4.7) as per manufacturers' protocol. Culture supernatant samples were brought to room temperature prior to analysis and assay standards were reconstituted in complete media. Standards and samples were analysed in duplicate. Immunoassay microplates were analysed on Luminex

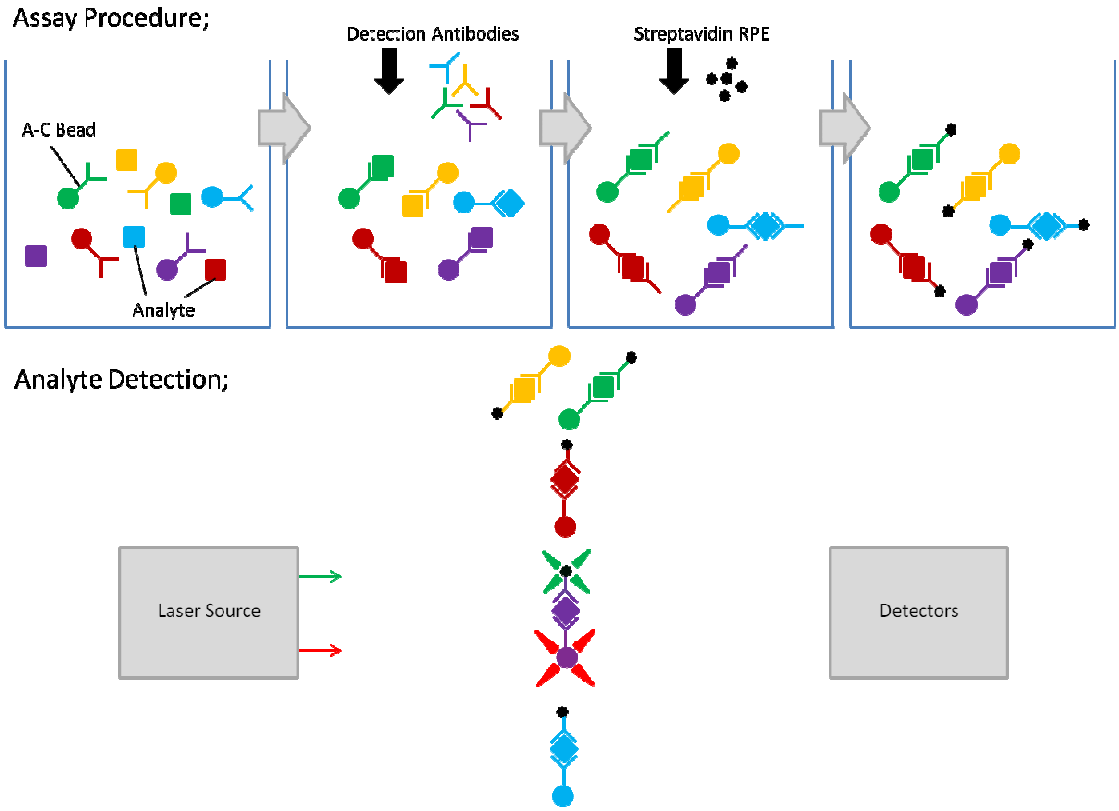


Figure 2-8 Schematic Representation of the Principle of Luminex Technology to Perform and Analyse Multiplex Bead Immunoassays

Assay procedure; Luminex multiplex bead immunoassays are performed in filter-bottom microplates. Each well contains a different analyte standard or test sample and is interrogated by incubation with beads conjugated to analyte-specific capture antibodies (A-C Beads). After washing, beads are incubated with analyte-specific biotinylated detector antibodies that bind the appropriate immobilised analyte. Following washing, the beads are incubated with Streptavidin conjugated to fluorescent R-Phycoerythrin (Streptavidin RPE) that binds the biotinylated detector antibodies associated with immune complexes on the beads.

Analyte detection; Assay microplates are analysed on Luminex 100 instrument. Beads from each well are streamed in single file past red and green lasers. The red laser excites bead internal red and infrared dyes allowing for bead identification. The green laser excites bound R-Phycoerythrin. Detectors monitor emissions from red and green laser excitation allowing for the identification of individual beads and quantification of bound analyte.

xMap Technology 100 machine with Luminex 100 Integrated System v2.3 software (Luminex Corporation). Luminex analysis was performed at the Transplant Immunology Department, St James's University Hospital, Leeds, UK.

2.6.2.1 Conditioned Media Samples

Conditioned media from HD9, HD23, HD54, HD85 and HD89 alginate cultures subjected to 48 hour treatment with IL-1β was selected for used in this study (Table 2-8).

2.6.2.2 Cytokine and Chemokine Targets

The commercially available human cytokine 10-plex and human chemokine 10-plex bead immunoassays were used in this study to facilitate simultaneous quantification of 20 targets (Table 2-10) in each conditioned media sample. Target cytokines and chemokines for these kits are shown in Table 2-10.

Cytokine 10-plex			Chemokine 10-plex		
Target	Sensitivity (pg/mL)	Correlation Coefficient	Target	Sensitivity (pg/mL)	Correlation Coefficient
IL-1β	<15	0.97	CCL2	<1	0.97
IL-2	<10	0.94	CCL3	<5	0.99
IL-4	<5	0.99	CCL4	<2	0.99
IL-5	<3	0.99	CCL5	<2	0.99
IL-6	<3	0.97	CCL7	<7	0.96
CXCL8	<10	0.97	CCL8	<2	0.98
IL-10	<5	0.93	CCL11	<1	0.95
CSF2	<15	0.99	CXCL1	<4	0.96
TNF-α	<10	0.92	CXCL9	<5	0.98
IFN-γ	<5	0.98	CXCL10	<1	0.97

Table 2-10 Details of Multiplex Bead Immunoassays used in Luminex Analysis of Conditioned Media from Alginate Nucleus Pulposus Cultures With and Without Interleukin-1β Stimulation

2.6.3 Analysis of Luminex Data

Luminex Integrated System produces a standard curve for each analyte investigated based on analysis of assay standards on each microplate. Sample data is expressed as a concentration reading in pg/mL for each analyte in each sample. Data was transferred to an Excel file where sample duplicate readings were averaged and then combined for each treatment group.

2.7 Statistical Analysis

Statistical analysis was applied to experimentally generated data following consultation with Dr Karen Kilner, Statistician, Sheffield Hallam University. In all cases, results were considered statistically significant if *P* values of less than 0.05 were recorded upon statistical analysis of experimentally generated data sets.

2.7.1 Statistical Analysis of Immunohistochemistry Data

2.7.1.1 Assessment of Percentage Immunopositivity in NP Tissue Sections from Non-degenerate, Degenerate and Infiltrated Study Groups

Percentage immunopositivity values were combined for non-degenerate, degenerate and infiltrated study groups. Data sets were examined by Shapiro-Wilkes test, to assess the probability that samples included in this study were derived from a population with a normal distribution. The results of Shapiro-Wilkes testing indicated that samples used were not derived from a population with a normal distribution ($P<0.05$). Accordingly, data were assessed by Kruskal-Wallis test, to identify significant differences in percentage immunopositivity across study groups. Where significant differences were indicated across study groups ($P<0.05$), Conover-Inman post hoc testing was applied to identify data sets that were significantly different, and to generate '*P*' values specific to that difference. Analysed data was represented graphically, by box and whisker plot of median immunopositivity and range values, using Prism v5 (GraphPad Software Inc.). Shapiro-Wilkes and Kruskal-Wallis statistical analysis was performed using StatsDirect software (StatsDirect Ltd.).

2.7.1.2 Assessment of Correlation between Percentage Immunopositivity and Histologically Determined Grade of Degeneration in Nucleus Pulposus Tissue Sections

Percentage immunopositivity values and histologically determined grades of degeneration were examined by Linear Regression analysis. Data was represented graphically by XY scatter plot with line of best fit. Graphical representations and Linear Regression Analysis was performed using Prism v5 (GraphPad Software Inc.).

2.7.2 Statistical Analysis of qPCR Low Density Array Data

2.7.2.1 Assessment of Relative Gene Expression Levels in Mildly Degenerate and Moderately Degenerate Study Groups

Relative gene expression values ($2^{-\Delta CT}$) were combined for mildly degenerate and moderately degenerate study groups. Data sets were examined by Shapiro-Wilkes test, to assess the probability that samples included in this study were derived from a population with a normal distribution. The results of Shapiro-Wilkes testing indicated that samples used were not derived from a population with a normal distribution ($P<0.05$). Accordingly, data were assessed by Mann Whitney test, to

identify significant differences in relative gene expression levels between study groups. Data was represented graphically by scatter plot and statistical analysis performed using Prism v5 (GraphPad Software Inc.).

2.7.2.2 Assessment of Frequency of Gene Expression Detection in Mildly Degenerate and Moderately Degenerate Study Groups

The proportion of samples where gene expression was detected was calculated for mildly degenerate and moderately degenerate study groups. Two-sample test of proportionality was used to determine whether the frequency of detection of any gene investigated was significantly different between mildly degenerate and moderately degenerate study groups, based only on the binomial distribution of presence or absence of expression. Two-sample test of proportionality was performed by the equation shown below to calculate a test statistic or 'z value'. Using a 95% confidence interval for a one-tailed test the significance value, *P*<0.05, is achieved for z values ≥1.65 (Currell & Dowman, 2005).

$$z = \frac{P_A - P_B}{\sqrt{P'(1-P') \times \left(\frac{1}{n_A} + \frac{1}{n_B}\right)}}$$

Where P is the experimentally measured proportion and hence the best estimate of the unknown true proportion for the population. A and B are the two study groups for comparison and n is the number of samples investigated. P' is a pooled value for the proportions of the two samples calculated using the equation:

$$P' = \frac{n_A P_A + n_B P_B}{n_A + n_B}$$

2.7.3 Statistical Analysis of qPCR Gene Expression Data from Directly Extracted Nucleus Pulposus Cells

2.7.3.1 Assessment of Relative Gene Expression Levels in Non-degenerate, Degenerate and Infiltrated Study Groups

Relative gene expression values ($2^{-\Delta CT}$) were combined for non-degenerate, degenerate and infiltrated study groups. Data sets were examined by Shapiro-Wilkes test, to assess the probability that samples included in this study were derived from a population with a normal distribution. The results of Shapiro-Wilkes testing indicated that samples used were not derived from a population with a normal distribution (*P*<0.05). Accordingly, data were assessed by Kruskal-Wallis test, to identify significant differences in relative gene expression across study groups. Where significant differences were indicated across study groups (*P*<0.05), Conover-Inman post hoc testing was applied to identify data sets that were significantly different, and to generate '*P*' values specific to that difference.

Analysed data was represented graphically, by scatter plot using Prism v5 (GraphPad Software Inc.). Shapiro-Wilkes and Kruskal-Wallis statistical analysis was performed using StatsDirect software (StatsDirect Ltd.).

2.7.3.2 Assessment of Frequency of Gene Expression Detection in Non-degenerate, Degenerate and Infiltrated Study Groups

Statistical analysis was performed as described previously in section 2.7.2.2.

2.7.4 Statistical Analysis of qPCR Gene Expression Data from Monolayer and Alginate Cultured Nucleus Pulposus Cells Subjected to Cytokine Stimulation

Relative gene expression values ($2^{-\Delta CT}$) were combined for untreated and treated study groups. Data sets were examined by Shapiro-Wilkes test, to assess the probability that samples included in this study were derived from a population with a normal distribution. The results of Shapiro-Wilkes testing indicated that samples used were not derived from a population with a normal distribution (P<0.05). Accordingly, data were assessed by Kruskal-Wallis test, to identify significant differences in relative gene expression across study groups. Where significant differences were indicated across study groups (P<0.05), Conover-Inman post hoc testing was applied to identify data sets that were significantly different, and to generate 'P' values specific to that difference. Analysed data was represented graphically, by histogram using Excel 2010 (Microsoft Corporation). Shapiro-Wilkes and Kruskal-Wallis statistical analysis was performed using StatsDirect software (StatsDirect Ltd.).

2.7.5 Statistical Analysis of Luminex Data

Protein concentration in conditioned media values were combined for untreated and treated study groups. Data sets were examined by Shapiro-Wilkes test, to assess the probability that samples included in this study were derived from a population with a normal distribution. The results of Shapiro-Wilkes testing indicated that samples used were not derived from a population with a normal distribution (P<0.05). Accordingly, data were assessed by Kruskal-Wallis test, to identify significant differences in protein concentration across study groups. Where significant differences were indicated across study groups (P<0.05), Conover-Inman post hoc testing was applied to identify data sets that were significantly different, and to generate 'P' values specific to that difference. Analysed data was represented graphically using Prism v5 (GraphPad Software Inc.). Shapiro-Wilkes and Kruskal-Wallis statistical analysis was performed using StatsDirect software (StatsDirect Ltd.).

3 The Cytokine and Chemokine Gene Expression Profile of Nucleus Pulposus Cells

3.1 Introduction

This chapter describes initial investigations to determine the cytokine and chemokine gene expression profile of human NP cells. The cells used were profiled immediately upon their isolation from fresh human NP tissue and attention was given to some 91 cytokine, chemokine, receptor, accessory protein and activating enzyme targets. Real-time qPCR LDA was used to investigate target gene expression relative to internal reference gene expression in samples collected from 6 tissue donors. Comparative analysis of generated data was performed against two parameters; the frequency that gene expression of targets was detected, and when detected, the level at which expression was measured.

3.1.1 Study Design

3.1.1.1 Samples

Three cDNA samples were selected for analysis from each of two study groups; mildly degenerate and moderately degenerate (Table 3-1).

Mildly Degenerate		Moderately Degenerate	
Samples Used;	**Average Grade**	**Samples Used;**	**Average Grade**
HD3 (S)	4.8	HD5 (S)	9.0
HD9 (S)	2.8	HD23 (S)	7.8
HD30 (PM)	1.9	HD36 (S)	6.7
Mean Ages;			
34 years (range 25 – 45)		32 years (range 29 – 33)	

Table 3-1 cDNA Samples from Directly Extracted Nucleus Pulposus Cells used in LDA qPCR Analysis

S, surgical; PM, post-mortem. Full details of samples used in this study can be found in Appendix 1 and details of histological grading of tissue samples can be found in Chapter 2, section 2.2.3.

3.1.2 Methods

Full details of methods used in this study are given in Chapter 2, sections;

- 2.1 Tissue Sample Processing
- 2.2 Histology
- 2.5 Quantitative Polymerase Chain Reaction

3.1.3 Specific Objectives

This chapter aimed to address the hypothesis that:

Cytokines and chemokines are expressed in the IVD and expression is increased during IVD degeneration.

The specific objectives were:

- To determine the cytokine and chemokine mRNA expression profile of NP cells derived from mildly and moderately degenerate tissue
- To identify differences in expression profile between mildly and moderately degenerate study groups
- To select differentially expressed cytokines and chemokines for inclusion in further studies
- To investigate the stability of internal reference gene expression in NP cells and to select appropriate controls for use in further real-time qPCR gene expression studies

3.2 Results

3.2.1 Cytokine mRNA Expression in Nucleus Pulposus Cells

3.2.1.1 The Interleukin-1 Family

Relative IL-1 family mRNA values in DE NP cells are shown in Figure 3-1A. The frequency and level of mRNA detection of the agonist IL-1α was increased in moderately degenerate samples compared to mildly degenerate however, this was not significant (P>0.05 for both parameters). The frequency of mRNA detection of the agonist IL-1β was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; P<0.05). IL-1β mRNA expression, relative to internal reference gene expression, was also increased in moderately degenerate samples compared to mildly degenerate, however this was not significant (P>0.05). mRNA expression of the signalling receptor for these ligands, IL-1RI, and the signalling receptor accessory protein, IL-1RAcP, was detected in all samples and at equivalent levels between study groups. mRNA expression of the decoy receptor, IL-1RII, the receptor antagonist, IL-1Ra and the converting enzyme, ICE, was detected only in moderately degenerate samples. Both IL-1RII and IL-1Ra were detected in 2/3 samples and ICE was detected in 3/3 samples, indicating significantly increased frequency of mRNA expression in moderately degenerate samples compared to mildly degenerate (z value = 1.73; P<0.05, z value = 1.73; P<0.05 and z value = 2.45; P<0.05 respectively).

3.2.1.2 IL-6, IL-7 & IL-11

Relative mRNA values in DE NP cells for the cytokines IL-6, IL-7, IL-11 and associated receptors are shown in Figure 3-1B. The frequency of mRNA detection for IL-6 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$). IL-6 mRNA expression, relative to internal reference gene expression, was also increased in moderately degenerate samples compared to mildly degenerate, however this was not significant ($P>0.05$). mRNA expression for the signalling receptor, IL-6R, and the signalling accessory protein, gp130, was detected in all samples and at equivalent levels between study groups. IL-7 mRNA expression was not detected in any sample investigated. IL-7R mRNA expression was detected in 5/6 samples investigated and at equivalent frequencies and relative expression levels between study groups. The frequency of mRNA detection of IL-11 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$) however relative expression levels were seen to be equivalent between groups. The frequency of detection and relative expression level of IL-11Rα mRNA was equivalent between groups.

3.2.1.3 IL-15, IL-16, IL-18 & IL-23

Relative mRNA values in DE NP cells for the IL-15, IL-16, IL-18, IL-23 and associated receptors are shown in Figure 3-1C. IL-15 mRNA was not detected in any DE NP sample investigated however, the signalling receptor IL-15Rα was detected in 5/6 samples. The frequency of mRNA detection and relative expression levels were equivalent between study groups. The frequency of mRNA detection of IL-16 was equivalent between study groups although mRNA expression, relative to internal reference gene expression, was decreased in moderately degenerate samples compared to mildly degenerate, although this was not significant ($P>0.05$). Signalling receptor, CD4 mRNA expression was detected in 5/6 samples investigated and at equivalent frequency and relative expression level between study groups.

The frequency of mRNA detection of IL-18 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$). IL-18 mRNA expression, relative to internal reference gene expression, was also increased in moderately degenerate samples compared to mildly degenerate, although this was not significant ($P>0.05$). The frequency of mRNA detection of one of the signalling receptors for this ligand, IL-18Rα, was significantly increased in moderately degenerate samples – 3/3, compared to mildly

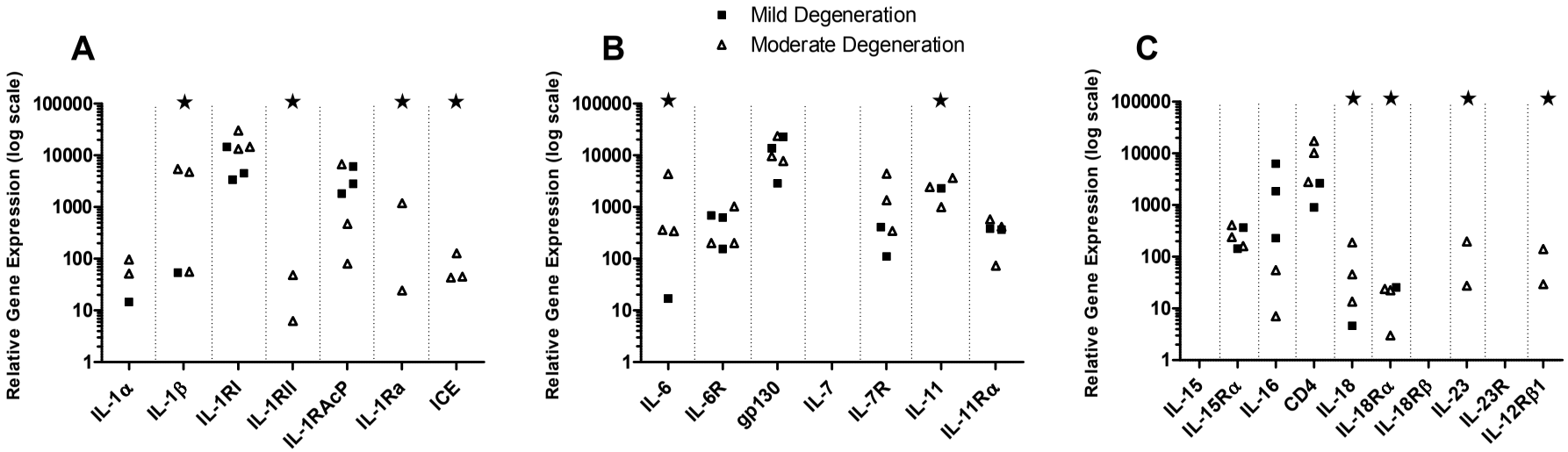


Figure 3-1 Cytokines; The IL-1 Family, IL-6, IL-7, IL-11, IL-15, IL-16, IL-18 and IL-23 mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Cytokine gene expression relative to internal reference gene expression in DE NP cells. In total, DE NP cells derived from six IVDs were investigated for each target gene. Graphical data represents measured relative mRNA expression in samples where expression was detected, i.e. for IL-1α; mRNA expression was detected in one mildly degenerate sample and in two moderately degenerate samples. ★ indicates significant alteration in frequency of mRNA detection between mildly and moderately degenerate study groups ($P<0.05$)

degenerate – 1/3 (z value = 1.73; *P*<0.05) although relative expression was equivalent between groups. mRNA expression of the second signalling receptor for this ligand, IL-18Rβ was not detected in any sample investigated.

The frequency of mRNA detection of the agonist IL-23 and one of the signalling receptors for this ligand, IL-12RβI was significantly increased in moderately degenerate samples – 2/3, compared to mildly degenerate – 0/3 (z value = 1.73; *P*<0.05 in both cases). mRNA expression of another signalling receptor for this ligand, IL-23R, was not detected in any sample investigated.

3.2.1.4 The IL-17 Family

Relative mRNA values in DE NP cells for the IL-17 family cytokines IL-17A, -B, -C, -D, -E and –F and associated receptors are shown in Figure 3-2A. mRNA expression of the agonists IL-17A and IL-17E was not detected in any DE NP sample investigated. mRNA expression of IL-17B, -C and –F was detected in only one of all samples investigated. The frequency of mRNA detection of the agonist IL-17D was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; *P*>0.05). IL-17D mRNA expression, relative to internal reference gene expression, was also increased in degenerate samples compared to non-degenerate, although this was not significant (*P*>0.05). The signalling receptors for these ligands, IL-17RA and IL-17RC, were detected in all samples investigated and at equivalent relative expression levels between study groups. The frequency of mRNA detection of the receptors, IL-17RB and IL-17RD, was equivalent between study groups although relative expression level was decreased in moderately degenerate samples compared to mildly, although this was not significant (*P*>0.05 in both cases).

3.2.1.5 IL-20

Relative mRNA values in DE NP cells for IL-20 and associated receptors are shown in Figure 3-2B. The frequency of mRNA detection and relative expression levels of the agonist IL-20, and signalling receptors, IL-20RII and IL-22RAI, was equivalent across study groups. mRNA expression of another signalling receptor for this ligand, IL-20RI was not detected in any sample investigated.

3.2.1.6 IL-4 and IL-10

Relative mRNA values in DE NP cells for the cytokines, IL-4 and IL-10 and associated receptors are shown in Figure 3-2C. mRNA expression of the agonist IL-4 was not detected in any DE NP sample investigated. The frequency of mRNA detection of the signalling receptor for this ligand, IL-4R was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z

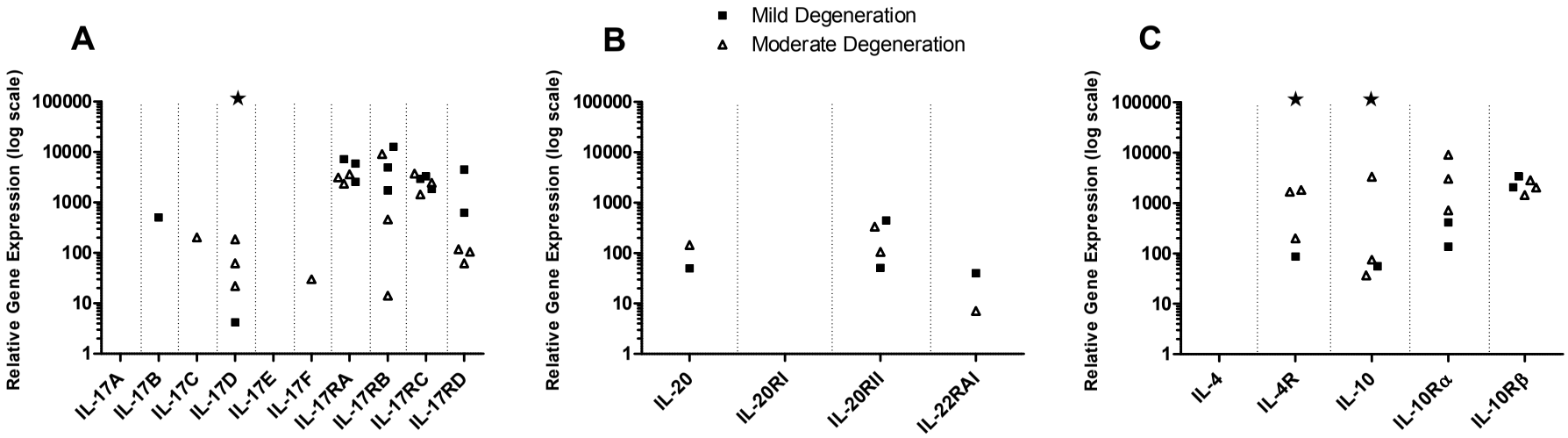


Figure 3-2 Cytokines; The IL-17 Family, IL-20, IL-4 and IL-10 mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Cytokine gene expression relative to internal reference gene expression in DE NP cells. In total, DE NP cells derived from six IVDs were investigated for each target gene. Graphical data represents measured relative mRNA expression in samples where expression was detected, i.e. for IL-17B; mRNA expression was detected only in one mildly degenerate sample. ★ indicates significant alteration in frequency of mRNA detection between mildly and moderately degenerate study groups (P<0.05)

value = 1.73; *P*<0.05). IL-4R mRNA expression, relative to internal reference gene expression, was also increased in moderately degenerate samples compared to mildly, although this was not significant (*P*>0.05). The frequency of mRNA detection of the agonist IL-10 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; *P*<0.05) although relative expression levels were equivalent between study groups. The frequency of mRNA detection of the signalling receptors for this ligand, IL-10Rα and IL-10Rβ, were equivalent between study groups, as was relative expression level of IL-10Rβ. Relative expression of IL-10Rα was increased in moderately degenerate samples compared to mildly, although this was not significant (*P*>0.05).

3.2.1.7 The TNF Family

Relative mRNA values in DE NP cells for the cytokines TNF-α and TNF-β, alongside associated receptors and converting enzyme are shown in Figure 3-3A. The frequency of mRNA detection of the agonist TNF-α was equivalent between study groups although mRNA expression, relative to internal reference gene expression, was increased in moderately degenerate samples compared to mildly, although this was not significant (*P*>0.05). The frequency of mRNA detection of the agonist TNF-β was equivalent in moderately degenerate – 1/3, and mildly degenerate – 1/3, samples. Expression of the signalling receptor, TNF-R55 and the activating enzyme, TACE was detected in all samples investigated; relative expression levels were equivalent between study groups. The frequency of mRNA detection of the signalling receptor, TNF-R75 was significantly increased in degenerate samples – 2/3, compared to non-degenerate – 0/3 (z value = 1.73; *P*<0.05).

3.2.1.8 CSF2, CSF3 and IFN-γ

Relative mRNA values in DE NP cells for the cytokines CSF2, CSF3 and IFN-γ alongside associated receptors are shown in Figure 3-3B. mRNA expression of CSF2 was only detected in 1/6 DE NP samples investigated. mRNA expression of CSF2Rα was detected only in moderately degenerate samples – 2/3, indicating significantly increased frequency of mRNA expression in moderately degenerate samples compared to mildly degenerate (z value = 1.73; *P*<0.05). The frequency of mRNA detection of signalling receptor, CSF2Rβ was equivalent between study groups as was relative expression level. Expression of the agonist CSF3 was not detected in any sample investigated. The frequency of mRNA detection of the receptor CSF3R was equivalent between study groups as was relative expression level. Expression of the agonist IFN-γ was not detected in any sample

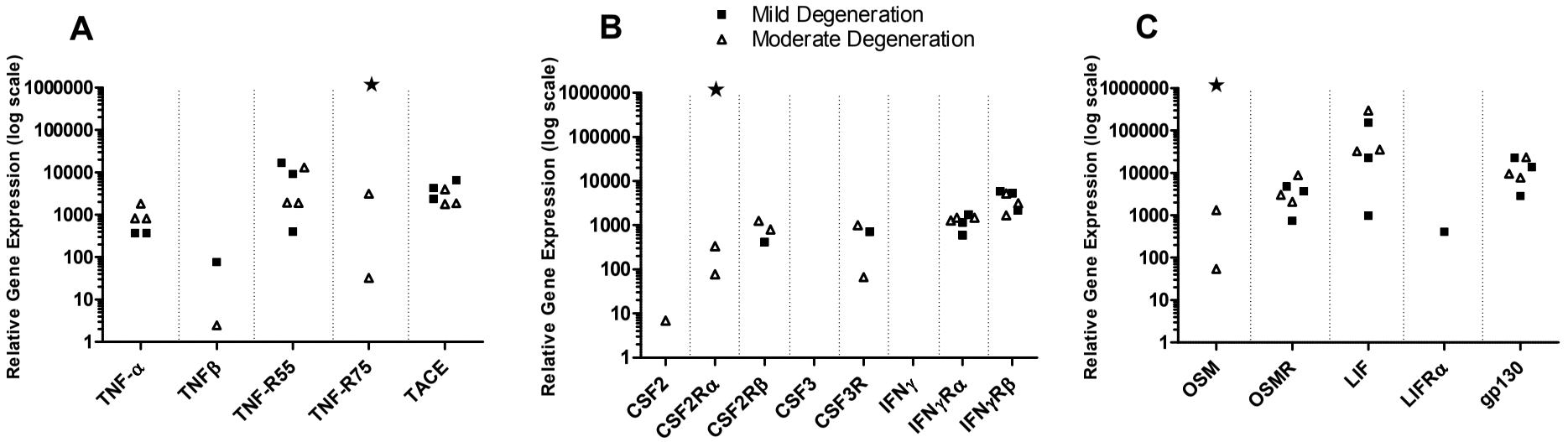


Figure 3-3 Cytokines; The TNF Family, CSF2, CSF3, IFN-γ, OSM and LIF mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Cytokine gene expression relative to internal reference gene expression in DE NP cells. In total, DE NP cells derived from six IVDs were investigated for each target gene. Graphical data represents measured relative mRNA expression in samples where expression was detected, i.e. for TNF-α; mRNA expression was detected in two mildly degenerate samples and in three moderately degenerate samples. ★ indicates significant alteration in frequency of mRNA detection between mildly and moderately degenerate study groups ($P<0.05$)

investigated. mRNA expression of the signalling receptors for this ligand, IFN-γRα and IFN-γRβ, were detected in all samples investigated and at equivalent relative expression levels between study groups.

3.2.1.9 OSM and LIF

Relative mRNA values in DE NP cells for the cytokines OSM and LIF alongside associated receptors and accessory protein are shown in Figure 3-3C. OSM mRNA expression was detected only in moderately degenerate samples indicating significantly increased frequency of expression in moderately degenerate samples – 2/3, compared to mildly – 0/3 (z value = 1.73; $P<0.05$). mRNA expression of the signalling receptor for this ligand, OSMR, was detected in all samples investigated and relative expression levels were equivalent between study groups. The frequency of mRNA detection of LIF was equivalent in moderately degenerate – 3/3, and mildly degenerate – 3/3, study groups as was relative expression level. mRNA expression of the signalling receptor for this ligand, LIFR was only detected in 1/6 DE NP samples investigated. Signalling receptor accessory protein, gp130 was detected in all samples as previously described (section 3.2.1.2).

3.2.2 Chemokine mRNA Expression in NP Cells

3.2.2.1 The C-C Chemokine Family

Relative mRNA values in DE NP cells for the chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL19 and CCL20 are shown in Figure 3-4A. The frequency of mRNA detection of CCL2 was equivalent in moderately degenerate samples – 3/3, compared to mildly degenerate – 2/3, although relative expression was increased in moderately compared to mildly degenerate samples, however this was not significant ($P>0.05$). The frequency of mRNA detection of CCL3 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$) and relative expression level was also increased, although this was not significant ($P>0.05$). The frequency of mRNA detection of CCL4 and CCL5 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 0/3 (z value = 2.45; $P<0.05$ in both cases). The frequency of mRNA detection of CCL7 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$) although relative expression levels were equivalent between groups. The frequency of mRNA detection of CCL8 was significantly increased in moderately degenerate samples – 2/3, compared to mildly degenerate – 0/3 (z value = 1.73; $P<0.05$). mRNA expression of CCL13 and CCL19 was only detected in 1/6 DE NP samples investigated. The frequency of mRNA detection of CCL20

was equivalent in moderately degenerate – 1/3, and mildly degenerate – 1/3, samples.

3.2.2.2 The C-X-C Chemokine Family and CX_3CL1

Relative mRNA values in DE NP cells for the chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10 and CX_3CL1 are shown in Figure 3-4B. The frequency of mRNA detection of CXCL1 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 1/3 (z value = 1.73; $P<0.05$). The frequency of mRNA detection of CXCL2 was equivalent between study groups; moderately degenerate – 3/3, compared to mildly degenerate – 2/3, although relative expression level was increased in moderately degenerate samples, however this was not significant ($P>0.05$). The frequency of mRNA detection of CXCL3 was significantly increased in moderately degenerate samples – 3/3, compared to mildly degenerate – 0/3 (z value = 2.45; $P<0.05$). The frequency of mRNA detection of CXCL5 was equivalent between study groups, moderately degenerate – 2/3, compared to mildly degenerate – 1/3. mRNA expression of CXCL6 was only detected in 1/6 DE NP samples investigated. The frequency of mRNA detection of CXCL8 was equivalent in moderately degenerate– 3/3, and mildly degenerate – 3/3, samples. CXCL8 mRNA expression, relative to internal reference gene expression, was increased in moderately degenerate samples compared to mildly degenerate, although this was not significant ($P>0.05$). The frequency of mRNA detection of CXCL9 was significantly increased in degenerate samples – 2/3, compared to non-degenerate – 0/3 (z value = 1.73; $P<0.05$). CXCL10 mRNA was not detected in any sample investigated. The frequency of mRNA detection of CX_3CL1 was equivalent in degenerate – 3/3, and non-degenerate – 3/3, samples as was relative expression level.

3.2.2.3 Chemokine Receptors

Relative mRNA values in DE NP samples for the chemokine receptors, CCR1, CCR2, CCR3, CCR5, CCR6, CCR7, CCR8, CXCR1, CXCR2, CXCR3, CX3CR1 and the decoy receptors Duffy and D6 are shown in Figure 3-4C. The frequency of mRNA detection of CCR1 was significantly increased in degenerate samples – 2/3, compared to non-degenerate – 0/3 (z value = 1.73; $P<0.05$). CCR2, CCR6 and CCR8 mRNA expression was not detected in any DE NP sample investigated. The frequency of mRNA detection of CCR3 was equivalent between study groups, moderately degenerate – 2/3, compared to mildly degenerate – 1/3. The frequency of mRNA detection of CCR5 was significantly increased in degenerate samples – 2/3, compared to non-degenerate – 0/3 (z value = 1.73; $P<0.05$).

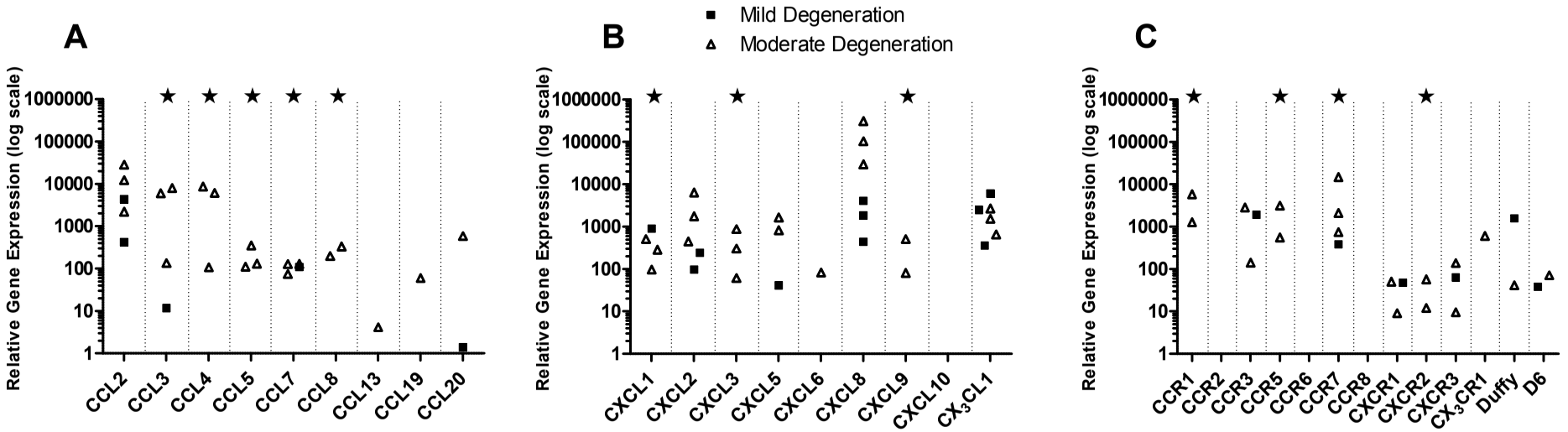


Figure 3-4 Chemokine mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Chemokine gene expression relative to internal reference gene expression in DE NP cells. In total, DE NP cells derived from six IVDs were investigated for each target gene. Graphical data represents measured relative mRNA expression in samples where expression was detected, i.e. for CCL2; mRNA expression was detected in two mildly degenerate samples and in two moderately degenerate samples. ★ indicates significant alteration in frequency of mRNA detection between mildly and moderately degenerate study groups (P<0.05)

The frequency of mRNA detection of receptors CXCR1 and CXCR3 was equivalent between study groups, moderately degenerate – 2/3, compared to mildly degenerate – 1/3. The frequency of mRNA detection of CXCR2 was significantly increased in degenerate samples – 2/3, compared to non-degenerate – 0/3 (z value = 1.73; $P<0.05$). mRNA expression of CX_3CR1 was only detected in 1/6 DE NP samples investigated. The frequency of mRNA detection of decoy receptors, Duffy and D6, was equivalent between study groups, moderately degenerate – 1/3, and mildly degenerate – 1/3, samples.

3.2.3 Summary of Results

The results of cytokine and chemokine ligand gene expression, presented in this chapter are summarised in Table 3-2 and Table 3-3.

3.3 Discussion

The objectives of this investigation were to determine the cytokine and chemokine gene expression profile of DE NP cells, and to identify potentially differentially expressed cytokines and chemokines between mildly and moderately degenerate study groups.

The data presented highlights transcription of numerous cytokines and chemokines by NP cells. Of the 18 cytokines detected, 6 are novel findings not previously reported in the IVD (IL-11, IL-16, IL-18, IL-23, OSM and LIF). Our findings, in relation to these targets within diseased IVD tissue agree with previous reports of their expression in OA and RA (Franz *et al.*, 1998; Klimiuk *et al.*, 1999; Abramson & Yazici, 2006; Andreas *et al.*, 2008; Sandell *et al.*, 2008; Moran *et al.*, 2009) (Table 1-4). Gene expression was not detected for 7 of the investigated cytokines (IL-7, IL-15, IL-17A, IL-17E, IL-4, CSF3 and IFN-γ) although some of these have been previously reported in the IVD. IL-17A and IL-17E gene expression was not detected in this study; however protein expression of IL-17 within the human IVD has been demonstrated previously by IHC (Shamji *et al.*, 2010). In the investigation by Shamji *et al*. (2010) the primary IL-17 antibody used was not isoform specific and hence protein detected may be translated from any of the six IL-17 family ligands, four of which (-B, -C, -D and –F) were identified here at the gene expression level. Also reported at the protein level in the human IVD are IL-4 and IFN-γ (Shamji *et al.*, 2010). Neither target was detected in this investigation however, in light of the small number of samples it's possible that the gene expression profiles of targets IL-17A, IL-17E, IL-4 and IFN-γ generated here are not representative of their expression in the wider population.

Target	Mildly Degenerate	Moderately Degenerate	Differential Expression Pattern
IL-1α	+	+	↑
IL-1β	+	+	↑*
IL-6	+	+	↑*
IL-7	-	-	
IL-11	+	+	↑*
IL-15	-	-	
IL-16	+	+	↓
IL-18	+	+	↑*
IL-23	-	+	↑*
IL-17A	-	-	
IL-17B	+	-	
IL-17C	-	+	
IL-17D	+	+	↑*
IL-17E	-	-	
IL-17F	-	+	
IL-20	+	+	
IL-4	-	-	
IL-10	+	+	↑*
TNF-α	+	+	↑
TNF-β	+	+	
CSF2	-	+	
CSF3	-	-	
IFNγ	-	-	
OSM	-	+	↑*
LIF	+	+	

Table 3-2 Summary of Cytokine mRNA Expression in Directly Extracted Nucleus Pulposus Cells from Mildly and Moderately Degenerate Study Groups and Observed Differential Expression Patterns

+, mRNA expression detected; -, mRNA expression not detected; ↑, increased in moderately degenerate study group in comparison to mildly degenerate study group; ↓ decreased in moderately degenerate study group compared to mildly degenerate study group, *, significant difference between frequency of mRNA detection between study groups ($P<0.05$)

Target	Mildly Degenerate	Moderately Degenerate	Differential Expression Pattern
CCL2	+	+	↑
CCL3	+	+	↑*
CCL4	-	+	↑*
CCL5	-	+	↑*
CCL7	+	+	↑*
CCL8	-	+	↑*
CCL13	-	+	
CCL19	-	+	
CCL20	+	+	
CXCL1	+	+	↑*
CXCL2	+	+	↑
CXCL3	-	+	↑*
CXCL5	+	+	
CXCL6	-	+	
CXCL8	+	+	↑
CXCL9	-	+	↑*
CXCL10	-	-	
CX_3CL1	+	+	

Table 3-3 Summary of Chemokine mRNA Expression in Directly Extracted Nucleus Pulposus Cells from Mildly and Moderately Degenerate Study Groups and Observed Differential Expression Patterns

+, mRNA expression detected; -, mRNA expression not detected; ↑, increased in moderately degenerate study group in comparison to mildly degenerate study group; ↓ decreased in moderately degenerate study group compared to mildly degenerate study group, *, significant difference between frequency of mRNA detection between study groups (P<0.05)

Of the 18 chemokine targets investigated, gene expression of 17 was detected in DE NP cells, 10 of which are novel findings not previously reported in the IVD (CCL3, CCL4, CCL8, CCL19, CCL20, CXCL2, CXCL3, CXCL5, CXCL6 and CX_3CL1). Our findings in relation to 9 of these targets within the diseased IVD agree with previous reports of their expression in OA and RA (Borzi *et al.*, 1999; Yuan *et al.*, 2001; Pulai *et al.*, 2005; Andreas *et al.*, 2008; Sandell *et al.*, 2008; Kelchtermans *et al.*, 2009) (Table 1-4). However, one other novel IVD chemokine, CCL19, has not previously been reported in OA or RA.

Gene expression of cytokine and chemokine receptors, signalling accessory molecules and activating enzymes were also considered. Frequently, receptor gene expression was detected alongside that of the ligand, indicating that autocrine or paracrine signalling mechanisms may function within NP tissue.

The major objective addressed in this chapter was the identification of cytokines and chemokines that are differentially expressed with increasing disease severity. Significant differences in transcription patterns between mildly and moderately degenerate study groups were apparent for several cytokines (Table 3-2) and chemokines (Table 3-3). However, data presented must be interpreted with caution. Firstly, because of the small number of tissue samples in each study group the data generated may not be representative of the wider population. Secondly, because five of the six tissue samples in this study are from prolapsed IVDs (protrusions constrained within the AF), the transcription patterns observed may be related to the pathology of prolapse rather than degeneration. And thirdly, since mRNA expression profiles may not be representative of expression profiles at a protein level. Nevertheless, data generated is useful to assist in the selection of cytokines and chemokines to be targeted for further investigation.

3.3.1 Target Selection for Further Gene Expression Studies

NP cytokines identified here were considered for inclusion in further gene expression studies initially based on differential expression patterns between mildly and moderately degenerate study groups. Targets were then prioritised according to detection of receptor mRNA transcription, since this may indicate the potential of cells to respond to signalling. Consideration was also given to current literature and documented links to IVD pathology. By these criteria, seven cytokines were selected for inclusion in further gene expression studies; IL-1β, IL-6, IL-16, IL-18, IL-17D, TNF-α and OSM (Table 3-4). An additional two cytokines were added to this selection; IL-20, which did not show a differential expression profile however, receptor transcription was detected and current literature indicates that this

Table 3-4 Selection of Cytokines for Further qPCR Analysis

(↑), mRNA expression increased in moderately degenerate study group in comparison to mildly degenerate study group; (↓) mRNA expression decreased in moderately degenerate study group compared to mildly degenerate study group; *, significant difference between frequency of mRNA detection between mildly and moderately degenerate study groups (P<0.05); R, mRNA expression of associated signalling receptor detected.

Target		Differential Expression Pattern	Receptor Expression	IVD Pathology	Further qPCR
IL-1α		(↑)	R	IVD degeneration and prolapse links (Ahn *et al.*, 2002; Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d)	
IL-1β		(↑)*	R	IVD degeneration links (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d)	✔
IL-6		(↑)*	R	IVD prolapse links (Kang *et al.*, 1996; Specchia *et al.*, 2002; Lee *et al.*, 2009)	✔
IL-7			R		
IL-11	Novel	(↑)*	R		
IL-15			R		
IL-16	Novel	(↓)	R		✔
IL-18	Novel	(↑)*	R*		✔
IL-23	Novel	(↑)*	R*		
IL-17A			R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	
IL-17B			R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	
IL-17C			R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	
IL-17D		(↑)*	R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	✔
IL-17E			R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	
IL-17F			R	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	

Target		Differential Expression Pattern	Receptor Expression	IVD Pathology	Further qPCR
IL-20			R	IVD prolapse links (Huang *et al.*, 2008)	✔
IL-4			R*	IVD degeneration links (Shamji *et al.*, 2010; Gabr *et al.*, 2011)	
IL-10		+ (↑)*	R	IVD prolapse links (Ahn *et al.*, 2002)	
TNF-α	+	+ (↑)	R*	IVD degeneration links (Weiler *et al.*, 2005; Le Maitre *et al.*, 2007d)	✔
TNF-β	Novel	+	R*		
CSF2	-	+	R*		
CSF3	-	-	R		
IFNγ	-	-	R		
OSM	Novel	+ (↑)*	R		✔
LIF	Novel	+	R		✔

Table 3-4 Continued from previous page.

Target		Differential Expression Pattern	Receptor Expression	IVD Pathology	Further qPCR
CCL2		(↑)	R (CCR1*/CCR2)	IVD prolapse links (Burke *et al*., 2002)	✔
CCL3	Novel	(↑)*	R (CCR1*/CCR5*)		✔
CCL4	Novel	(↑)*	R (CCR1*/CCR5*)		✔
CCL5		(↑)*	R (CCR1*/CCR3/CCR5*)	IVD prolapse link (Kawaguchi *et al*., 2002)	✔
CCL7		(↑)*	R (CCR1*/CCR2/CCR3/CCR5*)		✔
CCL8	Novel	(↑)*	R (CCR1*/CCR2/CCR3/CCR5*)		
CCL13			R (CCR1*/CCR2/CCR3/CCR5*)		
CCL19	Novel		R (CCR7*)		
CCL20	Novel				
CXCL1		(↑)*	R (CXCL2*)	IVD prolapse link (Kawaguchi *et al*., 2002)	✔
CXCL2	Novel	(↑)	R (CXCL2*)		✔
CXCL3	Novel	(↑)*	R (CXCL2*)		✔
CXCL5	Novel		R (CXCR1/CXCR2*)		
CXCL6	Novel		R (CXCR1)		
CXCL8		(↑)	R (CXCR1/CXCR2*)	IVD prolapse links (Burke *et al*., 2002)	✔
CXCL9		(↑)*	R (CXCR3)		
CXCL10			R (CXCR3)		
CX_3CL1	Novel		R (CX_3CR1)		✔

Table 3-5 Selection of Chemokines for Further qPCR Analysis

(↑), mRNA expression increased in moderately degenerate study group in comparison to mildly degenerate study group; (↓) mRNA expression decreased in moderately degenerate study group compared to mildly degenerate study group; *, significant difference between frequency of mRNA detection between mildly and moderately degenerate study groups ($P<0.05$); R, mRNA expression of associated signalling receptor detected.

cytokine has regulatory potential on other cytokines within the IVD (Huang *et al.*, 2008). And LIF, which also did not show a differential expression profile but was detected in all samples investigated and is a novel IVD cytokine. Selected cytokine targets are shown in Table 3-4.

Chemokines were selected for inclusion in further gene expression studies based on the criteria described above. However, priority was given to targets that signal through receptors CCR1 and CXCR2 as differential expression patterns were observed for these two molecules. 10 chemokines were selected as shown in Table 3-5.

3.3.2 Target Selection for Protein Expression and Localisation Studies

The gene expression profile may not be an accurate representation of molecules expressed by cells since intracellular regulatory mechanisms may function to prevent mRNA transcripts from being translated to proteins. Protein expression of cytokines is essential for them to function as extracellular signals and so must be confirmed before molecules can be implicated in cell biology. Cytokines from the panel selected for further mRNA expression studies were considered as potential targets for experimental work to confirm translation and to localise produced protein to cells of origin.

As expected, mRNA expression of both IL-1 agonists was detected in DE NP cells. Expression patterns were similar and transcription was increased in moderately compared to mildly degenerate samples, as has been shown previously (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d). Signalling receptor, IL-1RI, and receptor accessory protein, IL-1RAcP, transcription was detected in all samples indicating that NP cells are both producers and targets of IL-1 within the IVD. Interestingly, transcription of the antagonist, IL-1Ra, was significantly up-regulated in moderately compared to mildly degenerate samples as was that of the decoy receptor, IL-1RII, both of which function to dampen local IL-1 responses. Previously it has been demonstrated that IL-1Ra transcription is not up-regulated with increasing disease severity in the degenerate IVD (Le Maitre *et al.*, 2005) and this difference may be related to the pathology of prolapse rather than degeneration since tissue samples used here are both degenerate and prolapsed. Transcription of IL-1RII has not been shown previously in the IVD. IL-1β was selected for inclusion in further gene expression studies over IL-1α since the differential expression between study groups was significant for IL-1β. IL-1β was also selected for inclusion in further protein expression and localisation studies to facilitate comparison of data generated with that previously published. By this means it may be possible to

determine whether expression patterns are similar in degenerate NP tissue and prolapsed degenerate NP tissue. IL-1RI expression is well documented in the IVD (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d) and for this reason IL-1RI was not selected for further inclusion in gene or protein expression and localisation studies.

Transcription of IL-16 was detected in DE NP cells and data suggests that transcription may be decreased in line with increased disease severity. Further, transcription of the signalling receptor for this ligand, CD4 (Cruikshank *et al.*, 2000), was detected in DE NP cells indicating that NP cells have the capacity to respond to IL-16 signalling. Neither IL-16 nor CD4 have been described previously in the IVD. In fact, the finding of CD4 expression within DE NP cell samples may be considered unusual, since CD4 expression is normally only associated with T lymphocytes (Cruikshank *et al.*, 2000). However, since the cells used in this study were isolated from prolapsed tissue and leukocyte infiltration is a feature of prolapsed IVDs (Kanerva *et al.*, 1997; Habtemariam *et al.*, 1998; Kawaguchi *et al.*, 2002; Kobayashi *et al.*, 2009) in which T lymphocytes are identified as one population of infiltrating immune cells (Kanerva *et al.*, 1997; Habtemariam *et al.*, 1998) it is possible that the CD4 gene expression detected here is derived from contaminating T lymphocytes in the cell samples. Interestingly, CD4 gene expression was detected in all 5 of the DE NP cell samples derived from surgically obtained prolapsed IVD tissue but not in the DE NP cell sample derived from HD30, the only post-mortem non-degenerate sample included in the study. Since the normal IVD is considered an immune privileged site (Naylor, 1962; Bobechko & Hirsch, 1965), this observation may further indicate T lymphocyte contamination of surgically obtained prolapsed samples. As such, these two novel IVD targets were selected for inclusion in further gene expression studies and in protein expression and localisation studies to allow determination of the cellular source of IL-16 and CD4 within the IVD.

CCL2 and CXCL8 transcription was detected in DE NP cells and data indicates transcription of these two targets may be increased in line with increasing disease severity. CCL2 and CXCL8 have been identified previously in healthy and prolapsed IVD tissue, but not localised to the native IVD cells (Burke *et al.*, 2002). The study by Burke *et al*. (2002) reported expression of CCL2 in 40% of, and CXCL8 in 20% of, healthy IVD tissue samples indicating that physiological roles for CCL2 and CXCL8 within the normal IVD are likely. Both CCL2 and CXCL8 were selected for inclusion in further gene expression and protein expression studies.

CCL2 signals through receptors CCR1 and CCR2 (Murphy *et al.*, 2000). CCR1 transcription was detected in DE NP cells and transcription was increased in line with increasing disease severity. CCR2 transcription was not detected in any sample investigated however, in light of the small number of samples investigated this cannot be considered conclusive. Both receptors have been identified in articular chondrocytes (Silvestri *et al.*, 2003) and were selected as targets for inclusion in further gene expression studies. CCR1 was also selected for inclusion in protein expression and localisation studies.

CXCL8 signals through receptors CXCR1 and CXCR2 (Murphy *et al.*, 2000). Transcription of both receptors was detected in DE NP cells and data suggests CXCR2 transcription may be increased in line with increasing disease severity. Both receptors have been identified in articular chondrocytes (Silvestri *et al.*, 2003) and were selected for inclusion in further gene expression and protein expression and localisation studies.

CCL3 and CCL7 transcription was detected in DE NP cells. For both molecules differential transcription patterns indicate that transcription may be increased in line with increasing disease severity. CCL3 has not been documented previously in the IVD although expression by articular chondrocytes has been identified (Borzi *et al.*, 1999; Yuan *et al.*, 2001; Silvestri *et al.*, 2003; Sandell *et al.*, 2008). CCL7 gene expression has been reported previously in prolapsed IVDs (Kawaguchi *et al.*, 2002). Both chemokines signal through detected receptor CCR1 and were selected in preference to CCL4, CCL5 and CCL8 for inclusion in protein expression and localisation studies based on detection of mRNA transcripts in a greater proportion of samples investigated. Of the remaining C-C chemokines investigated here, CCL4 was also selected for inclusion in further protein studies since CCL5 mRNA expression has been investigated previously (Ahn *et al.*, 2002; Kawaguchi *et al.*, 2002) and CCL4 expression was detected at greater frequencies than that of CCL8. However, CCL4 and CCL5 were included alongside CCL3 and CCL7 in further gene expression studies.

3.3.3 Selection of Cytokine and Chemokine *In Vitro* Treatments

For continuity, cytokines and chemokines selected for investigation in protein expression and localisation studies were selected as treatments for *in vitro* experimental work.

3.3.4 Summary

Data presented in this chapter illustrates the diverse cytokine and chemokine gene expression profile of human NP cells and identifies expression of 16 cytokines and

chemokines not previously reported in the human IVD. Further, these preliminary investigations indicate that certain cytokines and chemokines may be differentially expressed according to severity of pathology in the IVD.

4 The Cytokine and Chemokine Gene Expression Profile of Nucleus Pulposus Cells derived from Non-degenerate, Degenerate and Infiltrated Intervertebral Discs

4.1 Introduction

This chapter describes further qPCR investigations to determine the cytokine, chemokine and associated receptor gene expression profile of human NP cells. Attention is given to some 30 cytokine, chemokine and receptor genes selected following analysis of LDA data discussed in Chapter 3, sections 3.3.1 and 0, summarised in Table 3-4 and Table 3-5. Real-time qPCR was used to investigate mRNA expression in NP cells derived from 30 additional tissue samples, combined with 5/6 of the original samples used in previous studies (insufficient sample remaining for inclusion of HD5). Comparative analysis of generated data was performed against two parameters; the frequency that gene expression of targets was detected, and when detected, the level at which expression was measured.

4.1.1 Study Design

This study was performed to address the limitations of previous experimental work in determining the cytokine and chemokine gene expression profile of human NP cells. By expanding the number of samples included in the study data generated should be more representative of the wider population. Further, refining the study groupings used may help to identify mRNA expression profiles associated with the different pathologies of degeneration and prolapse. In this study samples were allocated to one of three categories; non-degenerate, degenerate or infiltrated. The rationale for these groupings was based on the requirement to analyse separately data generated from tissue undergoing the process of spontaneous resorption. These samples were identified by the presence of infiltrating cells (leukocytes) that form part of the inflammatory and/or auto-immune mechanisms of spontaneous resorption (see Chapter 1, sections 1.3.2.1, 1.3.2.2 and 1.3.2.3). Separating out this data should allow for the more accurate determination of differential expression profiles based on histological evidence of degenerative tissue changes (see Chapter 2, section 2.2.3).

4.1.1.1 Samples

35 cDNA samples derived from NP cells isolated from human tissue were selected for analysis from non-degenerate, degenerate and infiltrated study groups (Table 4-1).

Non-Degenerate	Degenerate	Infiltrated
Samples Used;		
HD9 (S)	HD3 (S)	HD1 (S)
HD22 (S)	HD16 (S)	HD2 (S)
HD24 (S)	HD17 (S)	HD21 (S)
HD30 (PM)	HD33 (S)	HD23 (S)
HD31 (PM)	HD34 (S)	HD25 (S)

Non-Degenerate	Degenerate	Infiltrated
HD44 (S)	HD45 (S)	HD26 (S)
	HD46 (S)	HD36 (S)
	HD51 (S)	HD49 (S)
	HD54 (S)	HD50 (S)
	HD56 (S)	HD52 (S)
	HD57 (S)	HD53 (S)
	HD58 (S)	HD66 (S)
	HD59 (S)	HD79 (S)
	HD61 (S)	
	HD63 (S)	
	HD75 (S)	
Mean Ages (range);		
37 years (23-45)	38 years (23-52)	38 years (26-62)

Table 4-1 cDNA Samples from Directly Extracted Nucleus Pulposus Cells used in qPCR Gene Expression Studies

(S, Surgical; PM, Post Mortem). Full details of samples used in this study can be found in Appendix 1 and details of histological grading of tissue samples can be found in Chapter 2, section 2.2.3.

4.1.1.2 Target Genes

9 target cytokines and 10 target chemokines were selected for investigation (see Chapter 3, section 3.3). Associated receptors were also included in this study to investigate the potential of NP cells to respond to cytokine and chemokine signalling (Table 4-2).

Cytokines	& Receptors	Chemokines	& Receptors
IL-1β		CCL2	CCR1
IL-6	IL-6R & gp130	CCL3	CCR2
IL-16	CD4	CCL4	
IL-17D		CCL5	
IL-18		CCL7	
IL-20		CXCL1	CXCR1
TNF	TNF-R55 & TNF-R75	CXCL2	CXCR2
LIF	LIFR	CXCL3	
OSM		CXCL8	
		CX_3CL1	CX_3CR1

Table 4-2 Cytokine and Chemokine Target Genes Investigated by qPCR in Directly Extracted Nucleus Pulposus Cells

4.1.2 Methods

Full details of methods used in this study are given in Chapter 2, sections;

- 2.1 Tissue Sample Processing
- 2.2 Histology
- 2.5 Quantitative Polymerase Chain Reaction

4.1.3 Specific Objectives

This chapter aimed to address the hypothesis that:

Cytokine and chemokine expression is increased during degeneration and prolapse of the IVD.

The specific objectives were:

- To determine the cytokine and chemokine mRNA expression profile of NP cells derived from non-degenerate, degenerate and infiltrated human NP tissue
- To identify differences in mRNA expression profile between non-degenerate, degenerate and infiltrated study groups

4.2 Results

4.2.1 Cytokine mRNA Expression in Human Nucleus Pulposus Cells

4.2.1.1 The Interleukins; IL-1β, IL-6, IL-16, IL-17D, IL-18, IL-20

Relative IL-1β mRNA values for DE NP cells are shown in Figure 4-1A. IL-1β mRNA was detected in 1/6 non-degenerate, 6/16 degenerate and 9/13 infiltrated samples. The frequency of mRNA detection was increased in degenerate samples compared to non-degenerate, although this was not significant (P>0.05). The frequency of mRNA detection was significantly increased in infiltrated samples compared to both non-degenerate (z value = 2.13; P<0.05) and degenerate (z value = 1.70; P<0.05), and relative mRNA expression level was also increased in infiltrated samples compared to both non-degenerate and degenerate however, this parameter was not significant (P>0.05).

Relative IL-6 mRNA values for DE NP cells are shown in Figure 4-1B. IL-6 mRNA was detected in 3/6 non-degenerate, 9/16 degenerate and 9/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups. IL-6 relative mRNA expression level was equivalent in non-degenerate and degenerate samples, but increased in infiltrated. This increase was significant against degenerate (P=0.0014) samples, but not against non-degenerate (P>0.05).

Relative IL-6R mRNA values for DE NP cells are shown in Figure 4-1C. IL-6R mRNA was detected in 6/6 non-degenerate, 15/16 degenerate and 10/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups.

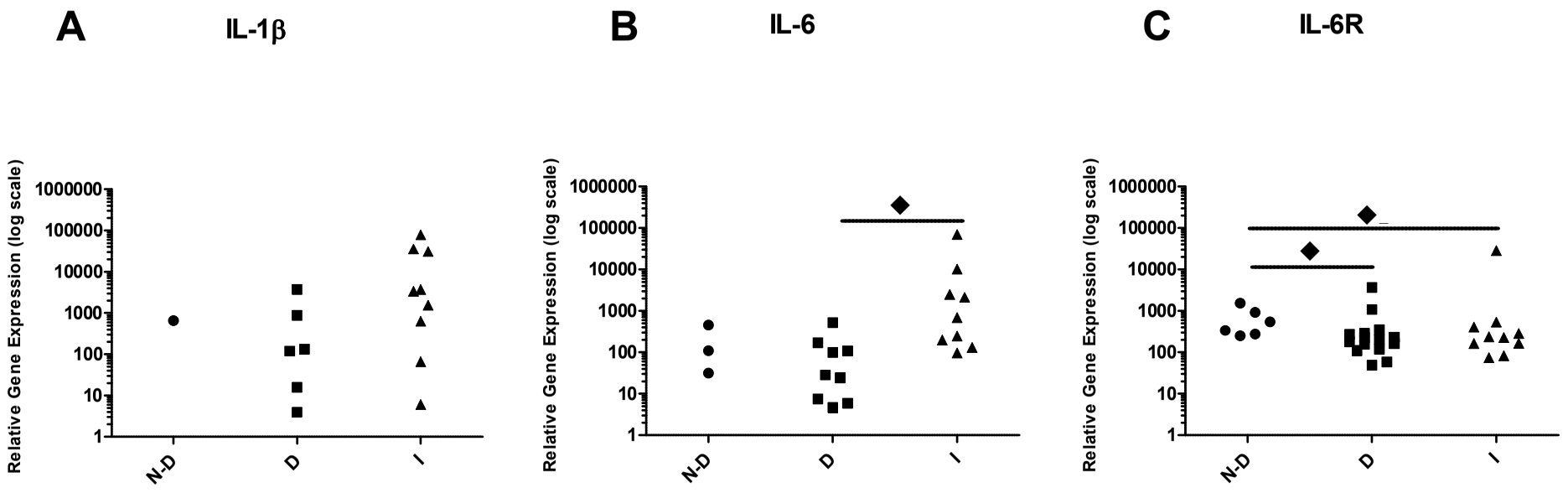


Figure 4-1 Relative IL-1β (A), IL-6 (B) and IL-6R (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for IL-1β; mRNA expression was detected in one out of six non-degenerate samples, in six out of sixteen degenerate samples and in nine out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups (P<0.05).

IL-6R relative mRNA expression level was significantly decreased in both degenerate and infiltrated samples compared to non-degenerate (P=0.0161 and P=0.0467 respectively). Relative expression levels were equivalent in degenerate and infiltrated samples.

Relative gp130 mRNA expression levels in DE NP cells are shown in Figure 4-2A. gp130 mRNA expression was detected in all samples investigated. Relative mRNA expression levels were equivalent in non-degenerate, degenerate and infiltrated samples.

Relative IL-16 mRNA expression in DE NP cells are shown in Figure 4-2B. IL-16 mRNA expression was detected in 6/6 non-degenerate, 15/16 degenerate and 11/13 infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent between study groups.

Relative CD4 mRNA expression in DE NP cells is shown in Figure 4-2C. CD4 mRNA expression was detected in 4/6 non-degenerate, 15/16 degenerate and 12/13 infiltrated samples. The frequency of mRNA detection was increased in both degenerate and infiltrated samples compared to non-degenerate, this difference was significant against degenerate (z value = 1.65; P<0.05), but not against infiltrated (P>0.05) samples. CD4 relative mRNA expression level was equivalent across study groups.

Relative IL-17D mRNA expression in DE NP cells is shown in Figure 4-3A. IL-17D mRNA expression was detected in 3/6 non-degenerate, 11/16 degenerate and 7/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups. IL-17D relative mRNA expression level was significantly decreased in degenerate samples compared to non-degenerate (P=0.0309). Relative mRNA expression levels were equivalent in non-degenerate and infiltrated samples.

Relative IL-18 mRNA expression in DE NP cells is shown in Figure 4-3B. IL-18 mRNA expression was detected in 2/6 non-degenerate, 1/16 degenerate and 3/13 infiltrated samples. The frequency of mRNA detection was decreased in degenerate samples compared to both non-degenerate and infiltrated samples, this was significant against non-degenerate (z value = 1.65; P<0.05), but not against infiltrated (P>0.05). The frequency of mRNA detection between non-degenerate and infiltrated samples was equivalent.

Relative IL-20 mRNA expression in DE NP cells is shown in Figure 4-3C. IL-20 mRNA expression was detected in 3/6 non-degenerate, 8/16 degenerate and 4/13

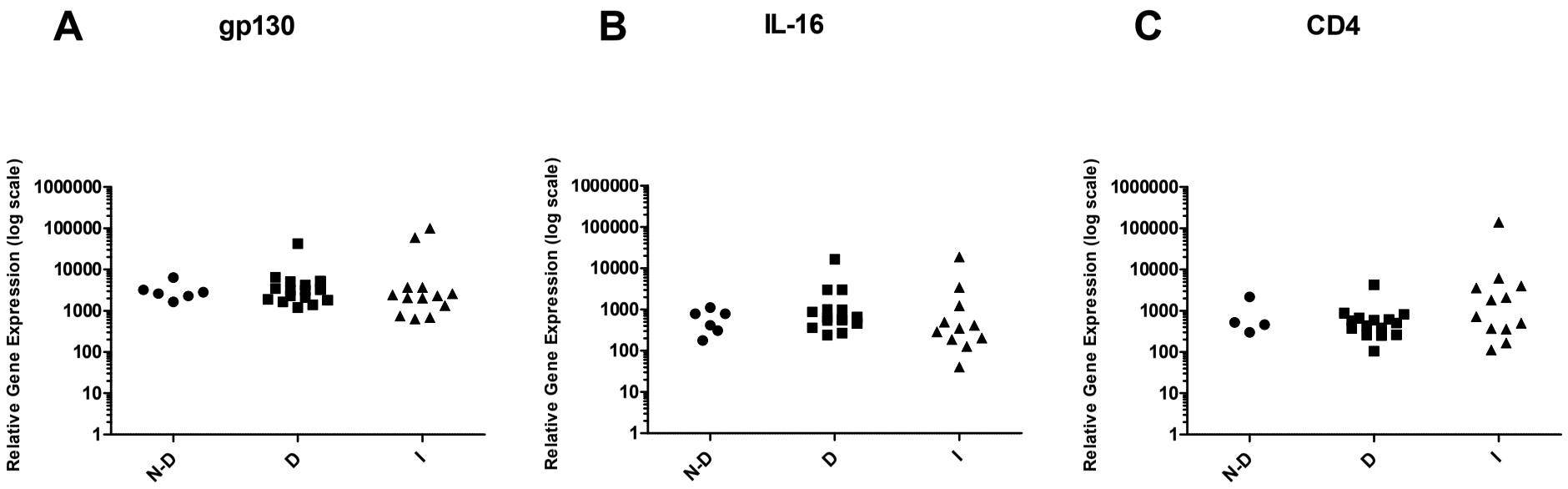


Figure 4-2 Relative gp130 (A), IL-16 (B) and CD4 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for gp130; mRNA expression was detected in six out of six non-degenerate samples, in sixteen out of sixteen degenerate samples and in thirteen out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

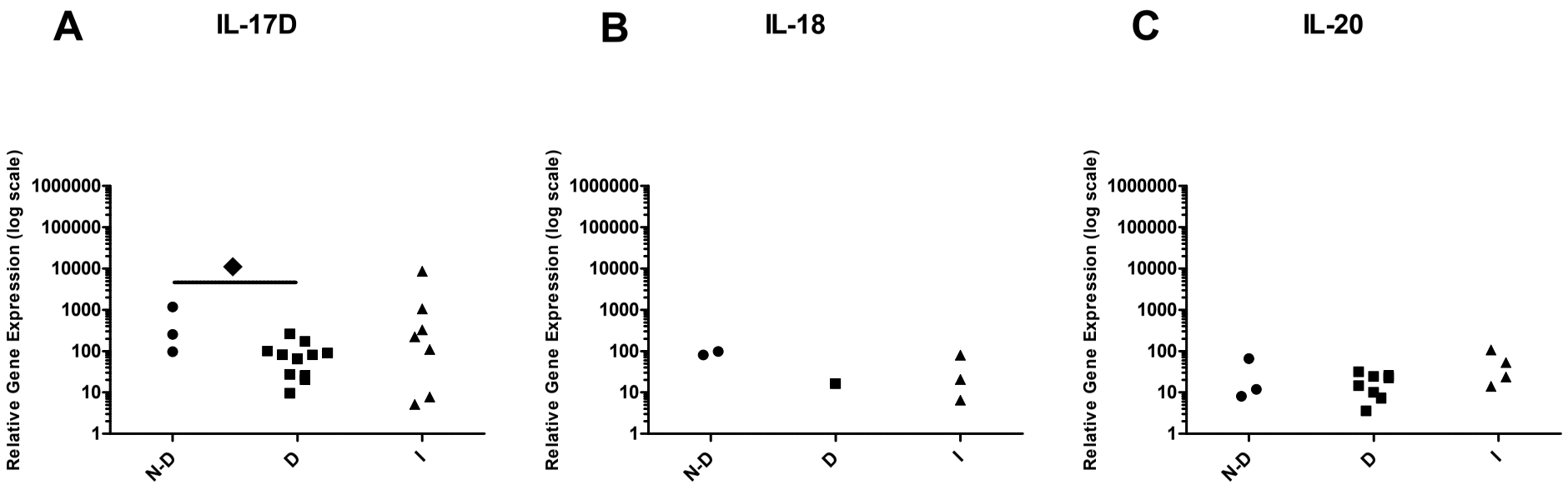


Figure 4-3 Relative IL-17D, IL-18 and IL-20 mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for IL-17D; mRNA expression was detected in three out of six non-degenerate samples, in eleven out of sixteen degenerate samples and in seven out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups (P<0.05).

infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent across study groups.

4.2.1.2 Other Cytokines; TNF, LIF, OSM

Relative TNF mRNA expression in DE NP cells is shown in Figure 4-4A. TNF mRNA expression was detected in 5/6 non-degenerate, 9/16 degenerate and 8/13 Infiltrated samples. The frequency of mRNA detection was decreased in both degenerate and infiltrated samples compared to non-degenerate although these differences were not significant (P>0.05). The frequency of mRNA detection was equivalent between degenerate and infiltrated samples. TNF relative mRNA expression level was equivalent in non-degenerate and degenerate samples, but increased in infiltrated. This increase was significant against degenerate samples (P=0.0028), but not non-degenerate (P>0.05).

Relative TNF-R55 mRNA expression in DE NP cells is shown in Figure 4-4B. TNF-R55 mRNA expression was detected in 6/6 non-degenerate, 15/16 degenerate and 10/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and degenerate samples and decreased in infiltrated although these differences were not significant (P>0.05). TNF-R55 relative mRNA expression level was increased in degenerate samples compared to both non-degenerate and infiltrated samples. This increase was significant against infiltrated samples (P=0.0092), but not against non-degenerate (P>0.05). Expression levels between non-degenerate and infiltrated samples were equivalent.

Relative TNF-R75 mRNA expression in DE NP cells is shown in Figure 4-4C. TNF-R75 mRNA expression was detected in 1/6 non-degenerate, 3/16 degenerate and 4/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups. TNF-R75 relative mRNA expression level was increased in infiltrated samples compared to degenerate (P=0.0286).

Relative LIF mRNA expression in DE NP cells is shown in Figure 4-5A. LIF mRNA was detected in 6/6 non-degenerate, 16/16 degenerate and 11/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups. LIF relative mRNA expression level was significantly decreased in degenerate samples compared to both non-degenerate (P=0.0301) and infiltrated samples (P=0.0256). mRNA expression levels were equivalent between non-degenerate and infiltrated samples.

Relative LIFR mRNA expression in DE NP cells is shown in Figure 4-5B. LIFR mRNA expression was detected in only 1/35 samples investigated.

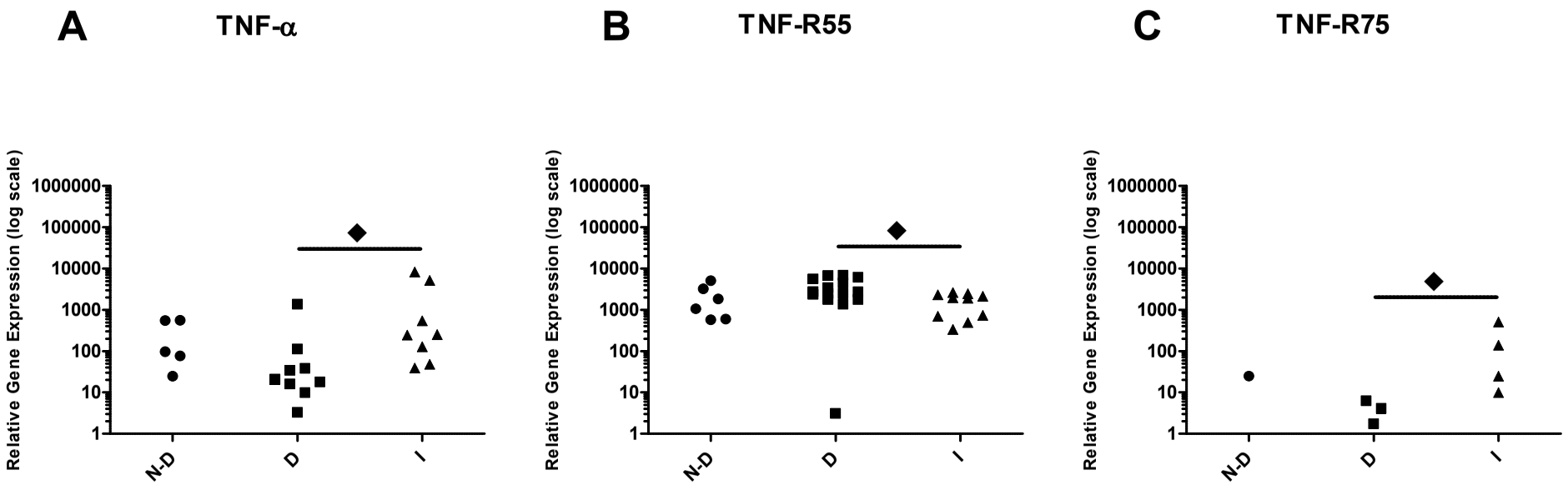


Figure 4-4 Relative TNF-α (A), TNF-R55 (B) and TNF-R75 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for TNF-α; mRNA expression was detected in five out of six non-degenerate samples, in nine out of sixteen degenerate samples and in eight out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

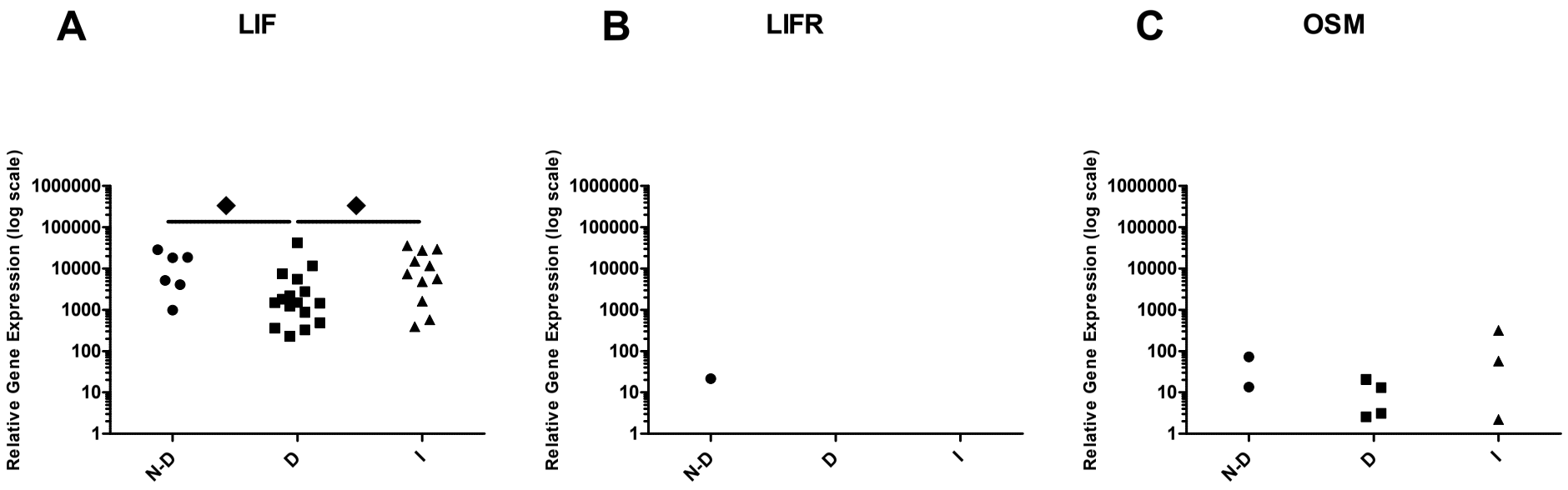


Figure 4-5 Relative LIF (A), LIFR (B) and OSM (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for LIF; mRNA expression was detected in six out of six non-degenerate samples, in sixteen out of sixteen degenerate samples and in eleven out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

Relative OSM mRNA expression in DE NP cells is shown in Figure 4-5C. OSM mRNA was detected in 2/6 non-degenerate, 4/16 degenerate and 3/13 infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent across study groups.

4.2.2 Chemokine mRNA Expression in Human NP Cells

4.2.2.1 C-C Chemokines; CCL2, CCL3, CCL4, CCL5, CCL7

Relative CCL2 mRNA expression in DE NP cells is shown in Figure 4-6A. CCL2 mRNA expression was detected in 5/6 non-degenerate, 13/16 degenerate and 13/13 infiltrated samples. The frequency of mRNA detection was equivalent in non-degenerate and degenerate samples but increased in infiltrated. This difference was significant against degenerate (z value = 1.65; $P<0.05$), but not against non-degenerate samples ($P>0.05$). CCL2 mRNA expression level was significantly decreased in degenerate samples compared to both non-degenerate ($P=0.0151$) and infiltrated ($P=0.0069$). Relative mRNA expression levels were equivalent in non-degenerate and infiltrated samples.

Relative CCL3 mRNA expression in DE NP cells is shown in Figure 4-6B. CCL3 mRNA expression was detected in 2/6 non-degenerate, 8/16 degenerate and 10/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and degenerate study groups but increased in infiltrated. This difference was significant against non-degenerate (z value = 1.83; $P<0.05$), but not against degenerate samples ($P>0.05$). CCL3 relative mRNA expression level was equivalent in non-degenerate and degenerate samples but increased in infiltrated. This difference was significant against degenerate ($P=0.0273$), but not against non-degenerate ($P>0.05$) samples.

Relative CCL4 mRNA expression in DE NP cells is shown in Figure 4-6C. CCL4 mRNA was detected in 2/6 non-degenerate, 5/16 degenerate and 9/13 infiltrated samples. The frequency of mRNA detection was equivalent in non-degenerate and degenerate samples but increased in infiltrated. This difference was significant against degenerate (z value = 2.04; $P<0.05$), but not against non-degenerate samples ($P>0.05$). CCL4 relative mRNA expression level was equivalent across study groups.

Relative CCL5 mRNA expression in DE NP cells is shown in Figure 4-7A. CCL5 mRNA was detected in 3/6 non-degenerate, 10/16 degenerate and 9/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and degenerate study groups but increased in infiltrated, although this difference was not significant. CCL5 relative mRNA expression level was

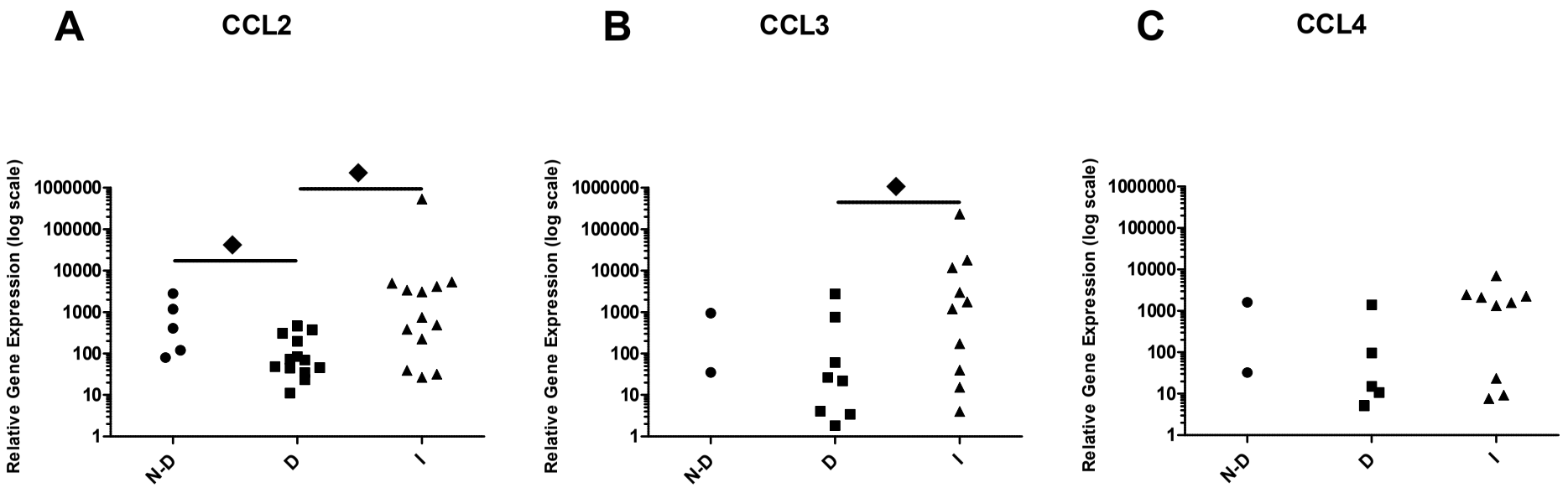


Figure 4-6 Relative CCL2 (A), CCL3 (B) and CCL4 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for CCL2; mRNA expression was detected in five out of six non-degenerate samples, in thirteen out of sixteen degenerate samples and in thirteen out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups (P<0.05).

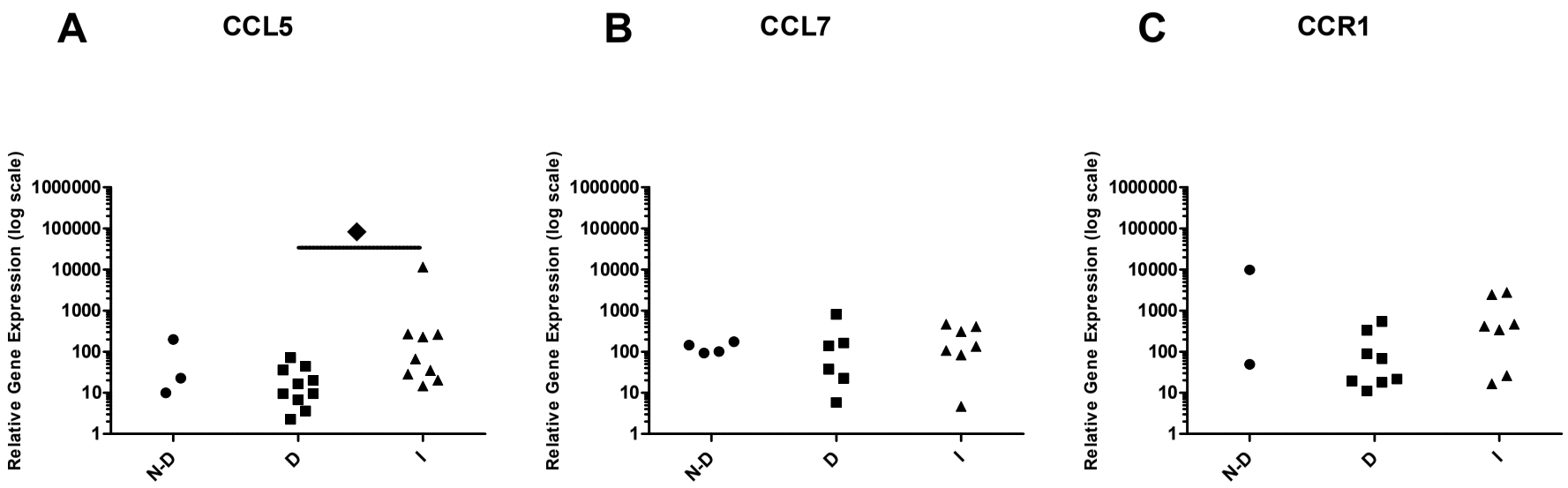


Figure 4-7 Relative CCL5 (A), CCL7 (B) and CCR1 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for CCL5; mRNA expression was detected in three out of six non-degenerate samples, in ten out of sixteen degenerate samples and in nine out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

equivalent between non-degenerate and degenerate samples but increased in infiltrated. This difference was significant against degenerate (*P*=0.0066), but not against non-degenerate samples (*P*>0.05).

Relative CCL7 mRNA expression in DE NP cells is shown in Figure 4-7B. CCL7 mRNA expression was detected in 4/6 non-degenerate, 6/16 degenerate and 7/13 infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent across study groups.

Relative CCR1 mRNA expression in DE NP cells is shown in Figure 4-7C. CCR1 mRNA expression was detected in 2/6 non-degenerate, 8/16 degenerate and 7/13 infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent across study groups.

CCR2 mRNA expression was not detected in any of the 35 DE NP samples investigated.

4.2.2.2 C-X-C Chemokines; CXCL1, CXCL2, CXCL3, CXCL8

Relative CXCL1 mRNA expression in DE NP cells is shown in Figure 4-8A. CXCL1 mRNA expression was detected in 4/6 non-degenerate, 7/16 degenerate and 8/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and infiltrated study groups but decreased in degenerate, although this difference was not significant. Relative mRNA expression levels were also equivalent between non-degenerate and infiltrated samples but decreased in degenerate. This difference was significant against infiltrated samples (*P*=0.0270) but not against non-degenerate (*P*>0.05).

Relative CXCL2 mRNA expression in DE NP cells is shown in Figure 4-8B. CXCL2 mRNA expression was detected in 5/6 non-degenerate, 11/16 degenerate and 9/13 infiltrated samples. The frequency of mRNA detection was equivalent across study groups. CXCL2 relative mRNA expression level was significantly decreased in degenerate samples compared to both non-degenerate (*P*=0.0207), and infiltrated (*P*=0.0061) samples. Relative mRNA expression levels were equivalent between non-degenerate and infiltrated samples.

Relative CXCL3 mRNA expression in DE NP cells is shown in Figure 4-8C. CXCL3 mRNA expression was detected in 4/6 non-degenerate, 7/16 degenerate and 8/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and infiltrated study groups but decreased in degenerate, although this difference was not significant. Relative mRNA expression levels were also equivalent between non-degenerate and infiltrated samples but decreased in

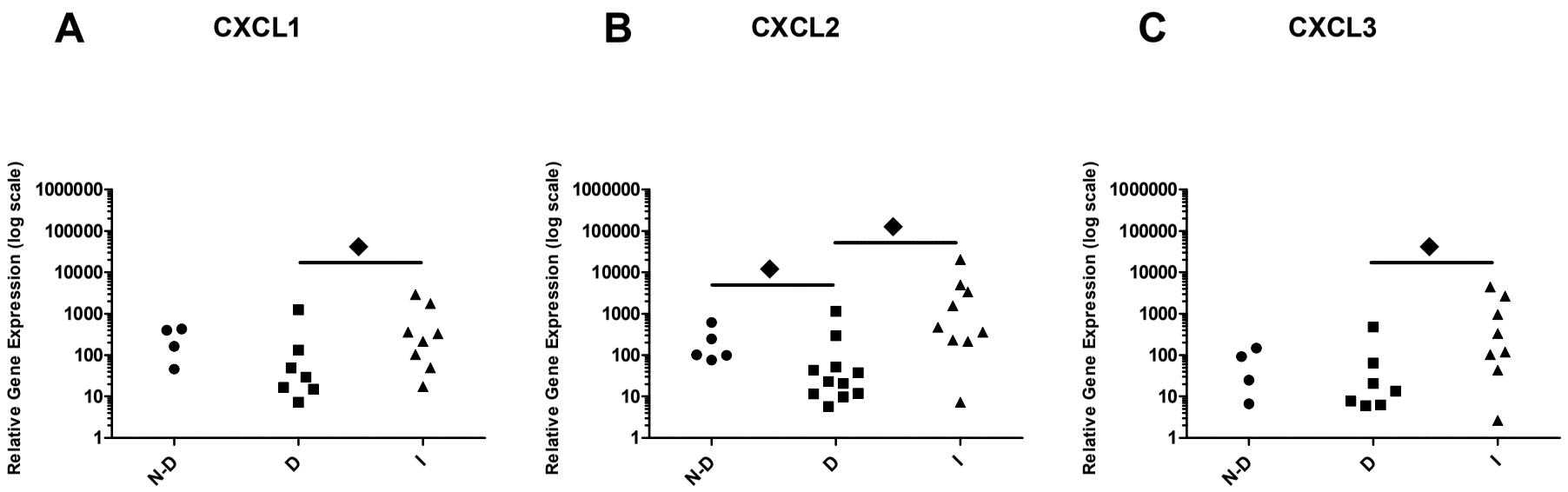


Figure 4-8 Relative CXCL1 (A), CXCL2 (B) and CXCL3 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for CXCL1; mRNA expression was detected in four out of six non-degenerate samples, in seven out of sixteen degenerate samples and in eight out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

degenerate. This difference was significant against infiltrated (*P*=0.0361), but not against non-degenerate (*P*>0.05) samples.

Relative CXCL8 mRNA expression in DE NP cells is shown in Figure 4-9A. CXCL8 mRNA expression was detected in 5/6 non-degenerate, 16/16 degenerate and 12/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and infiltrated samples but increased in degenerate. This difference was significant against non-degenerate samples (z value = 1.67; *P*<0.05), but not against infiltrated (*P*>0.05). CXCL8 relative mRNA expression level was equivalent in non-degenerate and degenerate samples but increased in infiltrated. This difference was significant against degenerate samples (*P*=0.0194), but not against non-degenerate (*P*>0.05).

Relative CXCR1 mRNA expression in DE NP cells is shown in Figure 4-9B. CXCR1 mRNA was detected in 1/6 non-degenerate, 3/16 degenerate and 4/13 infiltrated samples. The frequency of mRNA detection, and relative mRNA expression levels were equivalent across study groups.

Relative CXCR2 mRNA expression in DE NP cells is shown in Figure 4-9C. CXCR2 mRNA was detected in 0/6 non-degenerate, 3/16 degenerate and 5/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and degenerate study groups but increased in infiltrated. This difference was significant against non-degenerate samples (z value = 1.77; *P*<0.05), but not against degenerate (*P*>0.05). CXCR2 relative mRNA expression level was equivalent in degenerate and infiltrated samples.

4.2.2.3 C-X_3-C Chemokine; CX_3CL1

Relative CX_3CL1 mRNA expression in DE NP cells is shown in Figure 4-10A. CX_3CL1 mRNA expression was detected in 6/6 non-degenerate, 13/16 degenerate and 8/13 infiltrated samples. The frequency of mRNA detection was equivalent between non-degenerate and degenerate study groups but decreased in infiltrated. This difference was significant against non-degenerate samples (z value = 1.77; *P*<0.05) but not against degenerate (*P*>0.05). CX_3CL1 relative mRNA expression level was decreased in degenerate samples compared to both non-degenerate and infiltrated samples. This difference was significant against non-degenerate samples (*P*=0.0127) but not against infiltrated (*P*>0.05). Relative mRNA expression levels were equivalent in non-degenerate and infiltrated samples.

Relative CX_3CR1 mRNA expression in DE NP cells is shown in Figure 4-10B. CX_3CR1 mRNA expression was detected in 0/6 non-degenerate, 2/16 degenerate

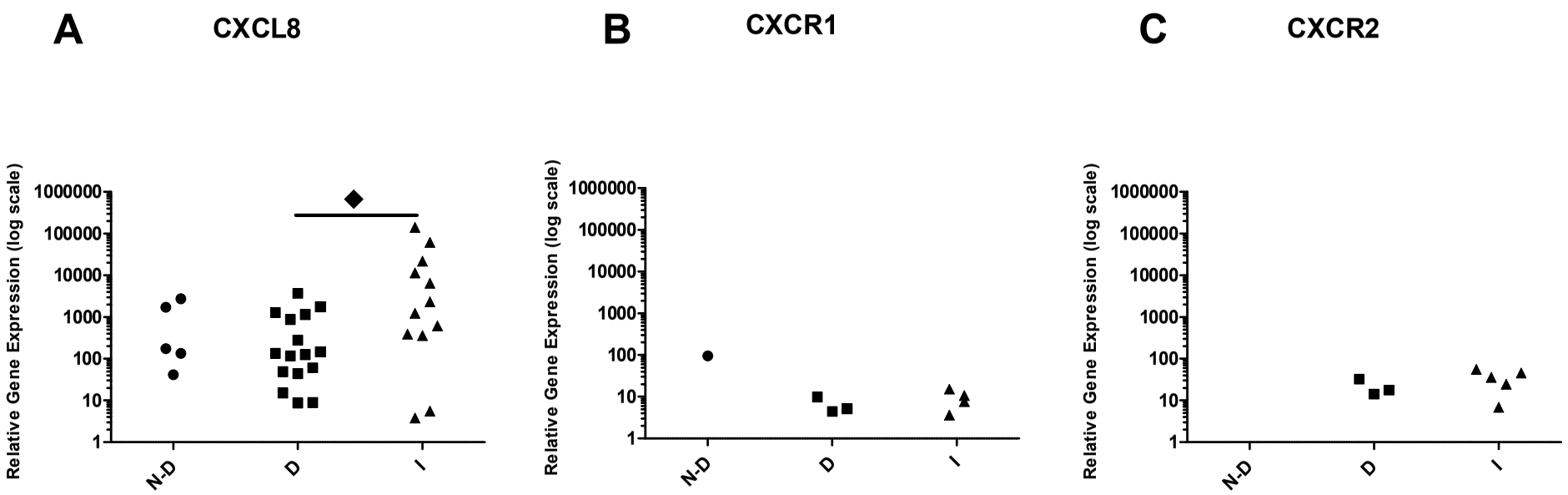


Figure 4-9 Relative CXCL8 (A), CXCR1 (B) and CXCR2 (C) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Study group, scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for CXCL8; mRNA expression was detected in five out of six non-degenerate samples, in sixteen out of sixteen degenerate samples and in twelve out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups (P<0.05).

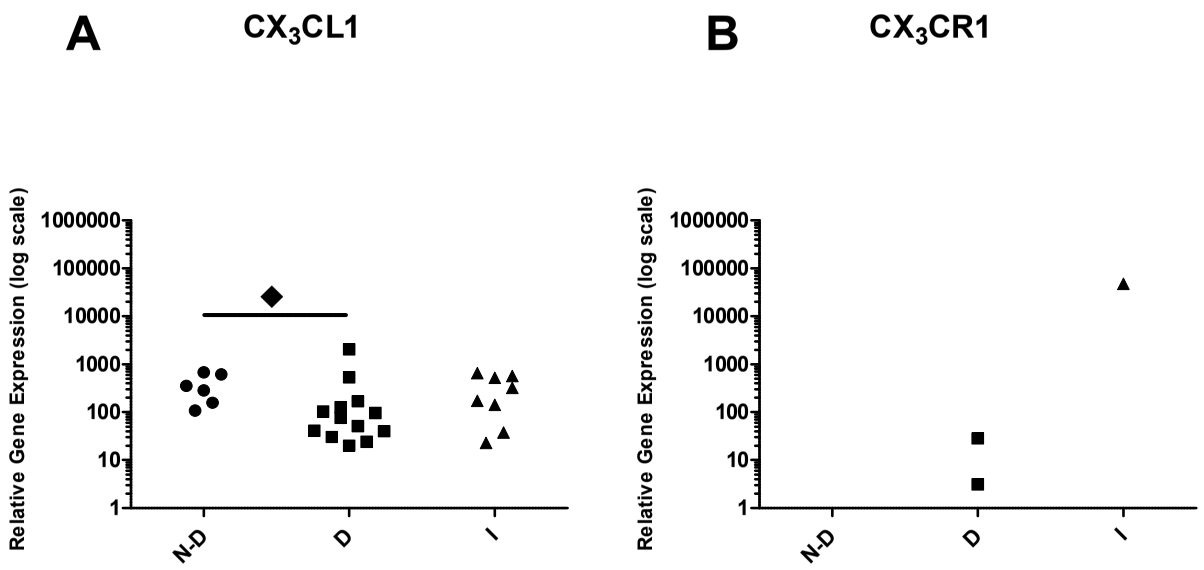


Figure 4-10 Relative CX_3CL1 (A) and CX_3CR1 (B) mRNA Expression in Directly Extracted Nucleus Pulposus Cells

Relative gene expression normalised to GAPDH and 18S internal reference genes. In total, DE NP cells derived from 35 IVDs were investigated for each target gene across three study groups; non-degenerate (n=6), degenerate (n=16) and infiltrated (n=13). Scatter plots represent measured relative mRNA expression in samples where expression was detected, i.e. for CX_3CL1; mRNA expression was detected in six out of six non-degenerate samples, in thirteen out of sixteen degenerate samples and in eight out of thirteen infiltrated samples. N-D, non-degenerate study group; D, degenerate study group; I, infiltrated study group; ◆, significant difference in relative mRNA expression level between study groups ($P<0.05$).

and 1/13 infiltrated samples. Comparative analysis of expression between study groups was not possible for this target due to low detection rates.

4.2.3 Summary of Results

The results presented in this chapter are summarised in Table 4-3 and Table 4-4.

Target	Non-Degenerate	Degenerate	Infiltrated	Altered mRNA Expression Level
IL-1β	1/6	6/16	9/13¤¥	
IL-6	3/6	9/16	9/13	↑¥
IL-6R	6/6	15/16	10/13	↓*↓¤
gp130	6/6	16/16	13/13	
IL-16	6/6	15/16	11/13	
CD4	4/6	15/16*	12/13	
IL-17D	3/6	11/16	7/13	↓*
IL-18	2/6	1/16*	3/13	
IL-20	3/6	8/16	4/13	
TNF-α	5/6	9/16	8/13	↑¥
TNF-R55	6/6	15/16	10/13	↓¥
TNF-R75	1/6	3/16	4/13	↑¥
LIF	6/6	16/16	11/13	↓*↑¥
LIFR	1/6	0/16	0/13	
OSM	2/6	4/16	3/13	

Table 4-3 Summary of Cytokine mRNA Expression in Directly Extracted Nucleus Pulposus Cells from Non-Degenerate, Degenerate and Infiltrated Study Groups

Numbers represent proportions of DE NP cell samples that were positive for mRNA expression. ↑, increase in relative mRNA expression level; ↓ decrease in relative mRNA expression level; *, significant difference between degenerate and non-degenerate study groups (P<0.05); ¤, significant difference between infiltrated and non-degenerate study groups (P<0.05); ¥, significant difference between infiltrated and degenerate study groups (P<0.05)

Target	Non-Degenerate	Degenerate	Infiltrated	Altered mRNA Expression Level
CCL2	5/6	13/16	13/13¥	↓*↑¥
CCL3	2/6	8/16	+10/13¤	↑¥
CCL4	2/6	15/16	9/13¥	
CCL5	3/6	10/16	9/13	↑¥
CCL7	4/6	6/16	7/13	
CCR1	2/6	8/16	7/13	
CCR2	0/6	0/16	0/13	
CXCL1	4/6	7/16	8/13	↑¥
CXCL2	5/6	11/16	9/13	↓*↑¥
CXCL3	4/6	7/16	8/13	↑¥
CXCL8	5/6	16/16*	12/13	↑¥
CXCR1	1/6	3/16	4/13	
CXCR2	0/6	3/16	5/13¤	
CX3CL1	6/6	13/16	8/13¤	↓*
CX3CR1	0/6	2/16	1/13	

Table 4-4 Summary of Chemokine mRNA Expression in Directly Extracted Nucleus Pulposus Cells from Non-Degenerate, Degenerate and Infiltrated Study Groups

Numbers represent proportions of DE NP cell samples that were positive for mRNA expression. ↑, increase in relative mRNA expression level; ↓ decrease in relative mRNA expression level; *, significant difference between degenerate and non-degenerate study groups (P<0.05); ¤, significant difference between infiltrated and non-degenerate study groups (P<0.05); ¥, significant difference between infiltrated and degenerate study groups (P<0.05)

4.3 Discussion

4.3.1 Cytokines

The objective of these investigations was to identify differentially expressed cytokines and chemokines in NP cells derived from non-degenerate, degenerate and infiltrated human NP tissue samples.

The non-degenerate study group was characterised by gene expression of numerous cytokines, particularly IL-6, IL-16, IL-17D, IL-20, TNF-α and LIF that were detected at high frequencies in DE NP cells derived from non-degenerate IVDs. However, gene expression of all targets investigated was confirmed in non-degenerate samples indicating a diverse cytokine expression profile. Where investigated, associated receptor gene expression was also confirmed in non-degenerate samples, with the exception of LIFR that was only detected in one sample (non-degenerate) of the 35 investigated, indicating a cytokine signalling network in these cells.

Gene expression of the same cytokines and receptors identified in the non-degenerate study group were confirmed in the degenerate, although the expression pattern was altered for some targets. Comparative analysis identified five cytokines with differential expression patterns; IL-1β, IL-17D, IL-18, TNF-α and LIF. IL-1β gene expression was not detected at high frequencies in non-degenerate or degenerate samples although a trend of up-regulated frequency was observed in degenerate samples. Up-regulation of IL-1β expression has been described previously in the degenerate IVD (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d) and whilst the findings here are not significant this may be due to the comparisons made being between prolapsed non-degenerate and prolapsed degenerate study groups, where non-prolapsed samples were used in previous investigations by Le Maitre *et al*. (2005 & 2007d). With the exception of IL-1β, differentially expressed cytokine targets were down-regulated in degeneration, IL-17D, IL-18 and LIF significantly so. This was unexpected and in some cases conflicted with data generated in previous preliminary investigations (see Chapter 3, section 3.2) where IL-17D and IL-18 expression was significantly up-regulated in moderately degenerate samples compared to mildly degenerate. IL-17D protein expression has been reported previously to be up-regulated in degenerate human IVDs and absent or present at very low concentration, in control IVD tissues (Shamji *et al.*, 2010). Here, gene expression was detected in 50% of non-degenerate samples and expression levels in degenerate samples were decreased in comparison. These differences may be accounted for by the gene expression profile observed here not being an accurate reflection of protein expression pattern, or may be

associated with the pathology of prolapse rather than degeneration, since non-prolapsed samples were used in studies by Shamji *et al*. (2010). IL-18 detection frequencies were low across all study groups, therefore whilst the down-regulation observed was statistically significant, in light of the low detection rates it is not possible to conclude that IL-18 down-regulation is a feature of degeneration. Our data indicates that TNF-α gene expression is down-regulated in degenerate IVDs compared to non-degenerate although this is not a significant finding. This conflicts with previous gene expression studies that have demonstrated up-regulation of TNF-α in the degenerate human IVD compared to the normal (Le Maitre *et al.*, 2007d; Tschoeke *et al.*, 2008) and with data generated in our previous preliminary investigation (Chapter 3, section 3.2.1.7) that indicated expression was up-regulated in line with increasing disease severity. Again this difference may be related to the use of prolapsed tissue in this study. Previously Ahn *et al*. (2002) investigated TNF-α mRNA expression in 23 prolapsed IVD tissue samples and reported 65% of samples investigated exhibited expression. Here, 33 of the 35 samples investigated were from prolapsed IVDs and 21 of these samples were positive for TNF-α mRNA, equating to 64% of samples investigated. Hence, our data confirms the previous report of TNF-α expression in prolapsed IVDs (Ahn *et al.*, 2002) and further, indicates that a differential expression profile exists within these samples dependent on degenerative tissue changes, but that this profile is not the same as the profile observed in non-prolapsed degenerate tissues.

LIF was identified in our previous preliminary investigation as a novel IVD cytokine (Chapter 3, section 3.2.1.9) and this is the first report of differential expression of this cytokine in non-degenerate and degenerate IVDs. LIF expression has been identified previously in human articular chondrocytes (Sandell *et al.*, 2008) and is found at elevated levels in the synovial fluid of RA sufferers (Okamoto *et al.*, 1997) although its role in the pathogenesis of RA is not well understood.

Comparative analysis of receptor gene expression between non-degenerate and degenerate study groups identified two differentially expressed target genes, IL-6R and CD4. These findings may indicate alterations in NP cell sensitivity toward the cytokines, IL-6 and IL-16 in degeneration. IL-6R expression was down-regulated whilst CD4 expression was up-regulated. IL-6R expression has been identified previously by IHC in prolapsed IVD tissue (Specchia *et al.*, 2002). Further, this study by Specchia *et al*. (2002) investigated IL-6R expression in non-degenerate, non-prolapsed IVD tissue and found that IL-6R was not expressed in these samples. In this study IL-6R gene expression was detected in all non-degenerate

samples investigated, two of which were derived from non-prolapsed, non-degenerate post-mortem IVD tissue. This would indicate that the receptor is expressed in non-degenerate IVDs. The ligand for this receptor, IL-6, was detected frequently in non-degenerate and degenerate IVDs (>50%) however, since receptor expression was down-regulated in degeneration NP cell sensitivity to this cytokine may be decreased in degeneration. Conversely, CD4 - the receptor for IL-16 (Cruikshank *et al.*, 2000) was up-regulated in degenerate samples compared to non-degenerate. IL-16 gene expression was detected frequently in non-degenerate and degenerate samples (>94%) and up-regulated receptor expression indicates increased NP cell sensitivity to this cytokine in degeneration. Both IL-16 and CD4 are novel IVD targets identified in our previous preliminary investigation (Chapter 3, section 3.2.1.3) and data generated in this study confirms our previous findings of expression by DE NP cells. In this investigation, our methodologies for sample categorisation were amended in order to exclude samples contaminated with infiltrating leukocytes from the non-degenerate and degenerate study groups (see Chapter 2, section 2.2.3.1). In light of the sample categorisation used here, data generated would indicate NP cell expression of CD4. However, whilst all non-degenerate samples were ligand (IL-16) positive only four of the six non-degenerate samples were receptor, CD4, positive. The two negative samples were non-degenerate, non-prolapsed post-mortem tissue and hence it remains to be resolved whether the CD4 gene expression observed here in surgical tissue samples relates to contaminating T lymphocyte expression, if perhaps the numbers of infiltrating cells are very small and imperceptible by our categorisation strategy.

Gene expression of the same cytokines and receptors identified in the non-degenerate study groups were confirmed in infiltrated. Comparative analysis identified few differences in target cytokine expression - only IL-1β was significantly altered between study groups. IL-1β mRNA expression was detected more frequently in infiltrated samples than in non-degenerate and degenerate samples although it's unknown from these investigations whether this increased detection is due to NP cell expression and potentially a factor in the mechanism of leukocyte recruitment or due to leukocyte expression of IL-1β. Investigations into IL-1β gene expression in the prolapsed IVD have reported 71% of samples investigated (n=9) were IL-1β mRNA positive (Le Maitre *et al.*, 2007d). This is comparable to our findings here in the infiltrated study group where 69% of samples investigated (n=11) were IL-1β positive, although overall we report a lower frequency of IL-1β detection in prolapsed samples with 16 out of the 33 investigated being mRNA

positive (48%). Interestingly, the study by Le Maitre *et al*. (2007d) investigated IL-1β mRNA expression in degenerate samples (n=22) and reported IL-1β expression in 100% of these whilst in non-degenerate samples expression was detected in 63% of samples investigated (n=8). Here only 38% of degenerate samples and 17% of non-degenerate samples were IL-1β mRNA positive.

Cytokine receptor gene expression was also similar between non-degenerate and infiltrated samples – only two differentially expressed cytokine receptor genes were identified; IL-6R and CD4. IL-6R gene expression was down-regulated in infiltrated samples similarly to the down-regulation observed in degenerate samples and again this may indicate decreased sensitivity to IL-6. CD4 gene expression was up-regulated in infiltrated samples similarly to the up-regulation observed in degenerate samples, although the increase was not significant in infiltrated samples. Again, this may indicate an increased sensitivity to IL-16 signalling in infiltrated samples. In contrast to comparative analysis between non-degenerate and degenerate study groups, IL-6 and TNF-α gene expression were up-regulated in infiltrated samples compared to non-degenerate.

4.3.2 Chemokines

Comparative analysis between non-degenerate and infiltrated study groups revealed a trend of up-regulated chemokine expression in infiltrated samples. The non-degenerate study group was characterised by gene expression of numerous chemokines, particularly CCL2, CCL5, CCL7, CXCL1, CXCL2, CXCL3, CXCL8 and CX_3CL1 that were detected at high frequencies in DE NP cells derived from non-degenerate IVDs. However, gene expression of all targets investigated was confirmed in non-degenerate samples indicating a diverse chemokine expression profile for these cells. Receptor gene expression was also confirmed in non-degenerate samples for CCR1 and CXCR1 indicating an active chemokine signalling network through these receptors in DE NP cells from the non-degenerate IVD.

Gene expression of the same chemokines and receptors identified in the non-degenerate study group was confirmed in the degenerate, alongside additional receptor gene expression detection for CXCR2 and CX_3CR1. Comparative analysis between non-degenerate and degenerate study groups revealed six differentially expressed chemokine targets; CCL2, CXCL1, CXCL2, CXCL3, CXCL8 and CX_3CL1. With the exception of CXCL8, differentially expressed chemokine targets were down-regulated in degeneration, CCL2, CXCL2 and CX_3CL1 significantly so. This was unexpected and in some cases conflicted with data

generated in previous preliminary investigations (see Chapter 3, section 3.23.2) where CXCL1 and CXCL3 expression was significantly up-regulated in NP cells from moderately degenerate samples compared to mildly degenerate.

CCL2 gene expression was detected at high frequencies in both non-degenerate and degenerate samples with decreased relative expression levels seen in degenerate samples. CCL2 protein detection has been reported previously in conditioned media from non-degenerate NP tissue explants (Burke *et al.*, 2002), where CCL2 protein was detected in media from 4 out of 10 (40%) explants investigated. Here, CCL2 gene expression was detected in 5 out of 6 (83%) non-degenerate samples investigated and this increase may be related to the non-degenerate samples used here being also prolapsed in 4 out of the 6 samples. CCL2 gene expression has been investigated previously in monolayer cultured primary human articular chondrocytes, where CCL2 gene expression was detected in cultures derived from OA sufferers but not from control specimens (Yuan *et al.*, 2001; Sandell *et al.*, 2008).

CXCL1 gene expression has been identified previously in prolapsed NP tissue (Kawaguchi *et al.*, 2002), CXCL2, CXCL3 and CX_3CL1 were identified in our previous preliminary investigation as novel IVD chemokines (Chapter 3, section 3.2.2.2). This is the first report of differential expression of these chemokines in non-degenerate and degenerate IVDs. CXCL1, CXCL2, CXCL3 and CX_3CL1 gene expression has been identified previously in cultured primary human chondrocytes derived from both normal articular cartilage and that of OA sufferers (Sandell *et al.*, 2008) with expression of CXCL1 and CXCL3 being increased in primary chondrocytes derived from OA sufferers compared to those from normal tissue donors (Sandell *et al.*, 2008). CXCL8 was the only chemokine that was up-regulated in degenerate samples compared to non-degenerate. CXCL8 protein production has been identified previously by ELISA in conditioned media from non-degenerate NP tissue explants (Burke *et al.*, 2002), where CXCL8 was produced by 2 out of 10 (20%) explants investigated. Here, CXCL8 gene expression was detected in 5 out of 6 (83%) non-degenerate samples and this increase may again be related to the pathology of prolapse rather than degeneration. CXCL8 gene expression has been identified in primary human articular chondrocytes and expression is increased in those derived from OA sufferers compared to normal tissue donors (Sandell *et al.*, 2008).

Gene expression of the same chemokines and receptors identified in the non-degenerate study group were confirmed in the infiltrated, alongside additional

receptor gene expression detection for CXCR2 and CX_3CR1. Comparative analysis between non-degenerate and infiltrated study groups revealed nine differentially expressed chemokine targets; CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL3, CXCL8 and CX_3CL1. With the exception of CX_3CL1 differentially expressed chemokine targets were up-regulated in infiltrated samples, although only CCL3 and CX_3CL1 expression was significantly altered compared to non-degenerate samples. CCL2 protein production has been identified previously by ELISA in the conditioned media of sequestered prolapsed NP tissue explants (Burke *et al.*, 2002), where CCL2 was detected in conditioned media from 8 out of 8 explants investigated. Here, CCL2 gene expression was detected similarly in all infiltrated samples investigated (13 out of 13), although this finding directly contradicts a previous investigation into CCL2 gene expression in prolapsed IVD tissue where 8 out of 8 tissue samples investigated were negative for CCL2 gene expression (Kawaguchi *et al.*, 2002). CCL3 and CCL4 were identified as novel IVD chemokines in previous preliminary investigations (see Chapter 3, section 3.2.2.1) and this is the first report of differential expression of these chemokines in non-degenerate, degenerate and infiltrated samples (Wang *et al*., 2012). CCL3 gene expression was detected in 10 out of 13 infiltrated samples (77%) and CCL4 in 9 out of 13 infiltrated samples (69%) although our report of CCL3 expression directly contradicts a previous study where 8 out of 8 prolapsed tissue samples investigated were negative for CCL3 gene expression (Kawaguchi *et al.*, 2002). CCL5 gene expression has been identified previously in prolapsed IVDs (Ahn *et al.*, 2002; Kawaguchi *et al.*, 2002), with reports of 4 out of 23 samples (17%) (Ahn *et al.*, 2002) and 7 out of 8 samples (88%) (Kawaguchi *et al.*, 2002) investigated being positive for CCL5 gene expression. In this study, 9 out of 13 (69%) infiltrated samples investigated were positive for CCL5 gene expression. CXCL1 gene expression has been identified previously in the prolapsed IVD (Kawaguchi *et al.*, 2002), where 5 out of 8 (63%) samples investigated were positive for CXCL1 gene expression. In this study, 8 out of 13 (62%) of infiltrated samples investigated were positive for CXCL1 gene expression. CXCL2 and CXCL3 were identified as novel IVD chemokines in previous preliminary investigations (see Chapter 3, section 3.2.2.2) and this is the first report of differential expression of these chemokines in non-degenerate, degenerate and infiltrated IVDs. CXCL8 gene expression has been identified previously in the prolapsed IVD (Ahn *et al.*, 2002), where 16 out of 23 (70%) samples investigated were positive for CXCL8 gene expression. Here, 12 out of 13 infiltrated samples (92%) investigated were positive for CXCL8 gene expression, although our report conflicts directly with a previous investigation into

CXCL8 gene expression in prolapsed NP tissue where 8 out of 8 samples investigated were CXCL8 negative (Kawaguchi *et al.*, 2002). CX_3CL1 was identified as a novel IVD chemokine in our previous preliminary investigation (see Chapter 3, section 3.2.2.2) and this is the first report of differential expression of this chemokine in non-degenerate, degenerate and infiltrated NP samples.

Again, data generated here must be interpreted with caution. Considering the non-degenerate study group, four of the six samples are derived from prolapsed IVDs. There have been several previous reports of increased cytokine and chemokine expression in prolapsed NP tissue (Kang *et al.*, 1996; Ahn *et al.*, 2002; Burke *et al.*, 2002; Kawaguchi *et al.*, 2002; Specchia *et al.*, 2002; Le Maitre *et al.*, 2007d; Shamji *et al.*, 2010) and so data generated here cannot be relied upon to be an accurate representation of the cytokine and chemokine expression profile of a non-degenerate IVD. Considering the degenerate study group, previous preliminary investigations (see Chapter 3, section 3.2) indicated a trend of cytokine and chemokine expression up-regulation with increasing severity of degenerative tissue changes. This was expected to translate here into a trend of up-regulation when considering degenerate samples to non-degenerate. However, this was not the case and may result from difficulties in classifying samples as either non-degenerate or degenerate. It was noted on histological examination of matched tissue sections that degenerative changes were not consistent throughout tissue samples but rather present as 'degenerative lesions' surrounded by histologically normal tissue. Upon this finding, multiple sections from each matched tissue sample were examined and corresponding cDNA samples were classified as 'degenerate' on the finding of degenerative lesions in any area of the tissue sample. Assuming that this variability was equivalent in adjacent tissue from which cell samples were extracted, it's impossible to know what percentage of cells derived from any tissue sample are from a degenerate lesion or a histologically normal tissue region. This factor may account for variability in expression levels of target cytokines and chemokines in the degenerate study group, and since data spread is a key parameter in statistical analysis this may directly affect the ability to identify significant differences in expression patterns.

Infiltrated samples were excluded from comparative analysis between non-degenerate and degenerate study groups on the basis of infiltration indicating active resorption. However, spontaneous resorption may not be the only feature of prolapse and so data generated in non-degenerate and degenerate study groups may be skewed on account of pathological changes related to prolapse rather than

degeneration. This scenario is reversed when considering the infiltrated study group, where variation in the severity of degenerative tissue changes between samples may account for some of the variation in observed expression levels. Whilst the expected up-regulatory trend of cytokine and chemokine expression was observed for the infiltrated study group, it remains unclear whether increased expression results from native NP cells or from the infiltrating leukocytes. In the case of IL-6, TNF-α, CCL3 and CXCL8, expression levels in infiltrated samples were increased in relation to all other samples. This would indicate that infiltration is a single factor associated with up-regulation, and provides evidence in support of contributing leukocyte production of these targets within the IVD.

Of note, upon histological examination of matched tissue sections, infiltrating cells were identified in tissue samples from intact IVDs (protrusion type of prolapse). This was unexpected as it was thought that AF/CEP rupture and exposure of NP tissue to the external environment of the IVD would need to occur to trigger leukocyte infiltration. One possible explanation for this finding is that NP tissue displaced down a radial fissure may eventually come into contact with the capillary network of the most outer AF. This would provide a direct route between blood circulation and NP tissue for circulating leukocyte entry into the intact IVD. Resolution without medical intervention has been described previously for protrusion type IVD prolapse, retraction of the AF is postulated to mediate this process (Guinto *et al.*, 1984; Teplick & Haskin, 1985). Based on our observations, leukocyte mediated breakdown of displaced NP tissue within the intact IVD could occur and may represent a mechanism in the process of protrusion resolution.

4.3.3 Summary

NP cells derived from prolapsed IVDs are characterised by an extensive and diverse cytokine, chemokine and receptor gene expression profile. Cells derived from degenerate prolapsed tissue exhibit significant differential expression patterns of cytokines and chemokines to those derived from non-degenerate prolapsed tissue; including down-regulation of IL-6R, IL-17D, LIF, CCL2, CXCL2 and CX_3CL1, and up-regulation of IL-1β, CD4, and CXCL8. Cells derived from infiltrated prolapsed tissue exhibit increased cytokine and chemokine gene expression compared to non-degenerate and degenerate prolapsed tissue including up-regulation of IL-1β, IL-6, TNF-α, CCL3 and CXCL8.

5 Cytokine and Chemokine Protein Production in the Intervertebral Disc

5.1 Introduction

This chapter describes investigations by IHC into the production and localisation of cytokines, chemokines and their receptors in human NP tissue. Further, microscopic evaluation of immunohistochemical and histological stains is used to determine the relationship between production of target molecules and the grade of degeneration in tissue samples.

Targets investigated are the cytokines; IL-1β and IL-16, the chemokines; CCL2, CCL3, CCL4, CCL7 and CXCL8, and the receptors; CD4, CCR1, CXCR1 and CXCR2 (see Chapter 3, section 0).

5.1.1 Study Design

5.1.1.1 Human Intervertebral Disc Tissue Samples

30 FFPE human IVD tissue sections were investigated for each target antigen, with the exception of IL-1β where 40 tissue sections were investigated. Sections for each target antigen were run together and alongside 15 matched isotype control sections (20 for IL-1β). For each IHC section investigated, an adjacent tissue section was stained with H&E for evaluation of degenerative tissue changes. Histological grades of adjacent sections for each IHC run are given in Table 5-1.

5.1.2 Methods

Full details of methods used in this study are given in Chapter 2, sections;

- 2.1 Tissue Sample Processing
- 2.2 Histology
- 2.3 Immunohistochemistry

5.1.3 Specific Objectives

This chapter aimed to address the hypothesis that:

Cytokines and chemokines are produced by the native cells of the IVD and production is increased during degeneration and prolapse of the IVD.

The specific objectives were:

- To confirm protein expression of target cytokines, chemokines and receptors in human NP tissue
- To localise protein expression of target cytokines, chemokines and receptors to cells of origin within human NP tissue
- To determine the relationship between production of target cytokines, chemokines and receptors and histologically determined grade of tissue degeneration

Table 5-1 Tissue Samples and Histological Grade of Degeneration for Sections Used in Immunohistochemistry Studies

S, surgical; PM, post-mortem; (i), infiltrated. For each tissue section used in immunohistochemistry studies an adjacent section was subject to histological staining and the grade of tissue degeneration assessed. Full details of samples used in this study can be found in Appendix 1**,** details of histological grading of tissue samples can be found in Chapter 2, section 2.2.3 and details of histological assessment of leukocyte infiltration can be found in Chapter 2, section 2.2.3.1.

Section	Immunohistochemistry Target										
	IL-1β	IL-16	CCL2	CCL3	CCL4	CCL7	CXCL8	CD4	CCR1	CXCR1	CXCR2
HD1 (S)	4.20 (i)	4.00 (i)	4.00 (i)	4.00 (i)	4.20 (i)	4.20 (i)	4.00 (i)	3.50 (i)	3.50 (i)	3.50 (i)	3.50 (i)
HD2 (S)	5.70 (i)	7.30 (i)	7.30 (i)	7.30 (i)	5.70 (i)	5.70 (i)	7.30 (i)	4.50 (i)	4.50 (i)	4.50 (i)	4.50 (i)
HD3 (S)	4.70	4.00	4.00	4.00	4.70	4.70	4.00	5.70	5.70	5.70	5.70
HD4 (S)	5.70 (i)	4.00 (i)	4.00 (i)	4.00 (i)	5.70 (i)	5.70 (i)	4.00 (i)	4.70 (i)	4.70 (i)	4.70 (i)	4.70 (i)
HD6 (S)	7.00 (i)	6.00 (i)	6.00 (i)	6.00 (i)	7.00 (i)	7.00 (i)	6.00 (i)	4.20 (i)	4.20 (i)	4.20 (i)	4.20 (i)
HD8 (S)	7.30	7.50	7.50	7.50	7.30	7.30	7.50	7.50	7.50	7.50	7.50
HD9 (S)	3.00	2.30	2.30	2.30	3.00	3.00	2.30	2.90	2.90	2.90	2.90
HD10 (S)			3.30	3.30	2.30		3.30				
HD11 (S)	7.50 (i)	7.50 (i)	7.50 (i)	7.50 (i)	7.50 (i)	7.50 (i)	7.50 (i)	3.50 (i)	3.50 (i)	3.50 (i)	3.50 (i)
HD12 (S)	5.50	7.40	7.40	7.40	5.50	5.50	7.40	6.20	6.20	6.20	6.20
HD14 (S)	5.50 (i)	5.70 (i)	5.70 (i)	5.70 (i)	5.50 (i)	5.50 (i)	5.70 (i)	4.50 (i)	4.50 (i)	4.50 (i)	4.50 (i)
HD15 (S)	7.50	6.20	6.20	6.20	7.50	7.50	6.20	9.20	9.20	9.20	9.20
HD30 (PM)	2.00	1.80	1.80	1.80	2.00	2.00	1.80	2.00	2.00	2.00	2.00
HD31 (PM)	2.50	3.00	3.00	3.00	2.50	2.50	3.00	3.50	3.50	3.50	3.50
HD32 (PM)	3.50	3.50	3.50	3.50	4.00	3.50	3.50	5.00	5.00	5.00	5.00

Section	Immunohistochemistry Target										
	IL-1β	IL-16	CCL2	CCL3	CCL4	CCL7	CXCL8	CD4	CCR1	CXCR1	CXCR2
HD33 (S)	6.00	9.50	9.50	9.50	6.00	6.00	9.50	8.40	8.40	8.40	8.40
HD34 (S)	3.50	11.00	11.00	11.00	3.50	3.50	11.00	3.90	3.90	3.90	3.90
HD36 (S)	6.50 (i)	8.50 (i)	8.50 (i)	8.50 (i)	6.50 (i)	6.50 (i)	8.50 (i)	4.50 (i)	7.40 (i)	4.50 (i)	4.50 (i)
HD37 (PM)	6.00	10.00	10.00	10.00	6.00	6.00	10.00	5.50	5.50	5.50	5.50
HD38 (PM)	6.00	8.00	8.00	8.00	6.00	6.00	8.00	8.50	8.50	8.50	8.50
HD39 (PM)	9.00	7.00	7.00	7.00	9.00	9.00	7.00	8.00	8.00	8.00	8.00
HD40 (PM)	7.30	8.00	8.00	8.00	7.30	7.30	8.00	8.50	8.50	8.50	8.50
HD41 (PM)	10.30	11.00	11.00	11.00	10.30	10.30	11.00	9.00	9.00	9.00	9.00
HD45 (S)	4.00	8.00	8.00	8.00	4.00	4.00	8.00	5.90	5.90	5.90	5.90
HD46 (S)		7.50				7.50					
HD49 (S)	4.70 (i)	6.00 (i)	6.00 (i)	6.00 (i)	4.70 (i)	4.70 (i)	6.00 (i)	5.00 (i)	5.00 (i)	5.00 (i)	5.00 (i)
HD50 (S)	2.30 (i)	3.00 (i)	3.00 (i)	3.00 (i)	2.30 (i)	2.30 (i)	3.00 (i)	4.40 (i)	4.40 (i)	4.40 (i)	4.40 (i)
HD51 (S)	7.00	10.50	10.50	10.50	7.00	7.00	10.50	5.40	5.40	5.40	5.40
HD52 (S)	6.20 (i)	9.00 (i)	9.00 (i)	9.00 (i)	6.20 (i)	6.20 (i)	9.00 (i)	4.00 (i)	4.00 (i)	4.00 (i)	4.00 (i)
HD53 (S)	5.20 (i)	5.80 (i)	5.80 (i)	5.80 (i)	5.20 (i)	5.20 (i)	5.80 (i)	4.50 (i)	4.50 (i)	4.50 (i)	4.50 (i)
HD54 (S)	3.50	7.70	7.70	7.70	3.50	3.50	7.70	7.40	7.40	7.40	7.40
HD56 (S)	6.00										
HD58 (S)	6.00										
HD59 (S)	7.00										
HD60 (S)	7.00 (i)										
HD61 (S)	6.00										

Table 5-1 Continued from previous page.

Section	Immunohistochemistry Target										
	IL-1β	IL-16	CCL2	CCL3	CCL4	CCL7	CXCL8	CD4	CCR1	CXCR1	CXCR2
HD63 (S)	9.00										
HD64 (S)	5.00 (i)										
HD71 (S)	4.00 (i)										
HD73 (S)	8.00										
HD74 (S)	4.00										
HD75 (S)	7.00										
HD85 (S)										5.40 (i)	
HD89 (S)								5.20	5.20		5.20
n =	40	30	30	30	30	30	30	30	30	30	30

Table 5-1 Continued from previous page.

5.2 Results

5.2.1 Cytokine Expression in Nucleus Pulposus Tissue

5.2.1.1 IL-1β Expression

IL-1β immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=6) degenerate (n=20), and infiltrated (n=14) IVDs. NP cell immunopositivity was observed in all tissue sections. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-1).

IL-1β immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-2A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 32.94%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 45.33%, and infiltrated IVDs, median 46.08%, although these differences were not significant (P>0.05). Regression analysis considering IL-1β immunopositivity and histologically determined grade of degeneration confirms that no linear relationship exists between IL-1β immunopositivity and grade of degeneration in tissue sections from human IVDs (P>0.05; Figure 5-2B).

5.2.1.2 IL-16 Expression

IL-16 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=4) degenerate (n=15), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in all tissue sections with the exception of 1 non-degenerate. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-3).

IL-16 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-4A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 28.87%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 36.10%, and infiltrated IVDs, median 35.15%, although these differences were not significant (P>0.05). Regression analysis considering IL-16 immunopositivity and histologically determined grade of degeneration confirms that no linear relationship exists between IL-16 immunopositivity and grade of degeneration in tissue sections from human IVDs (P>0.05; Figure 5-4B).

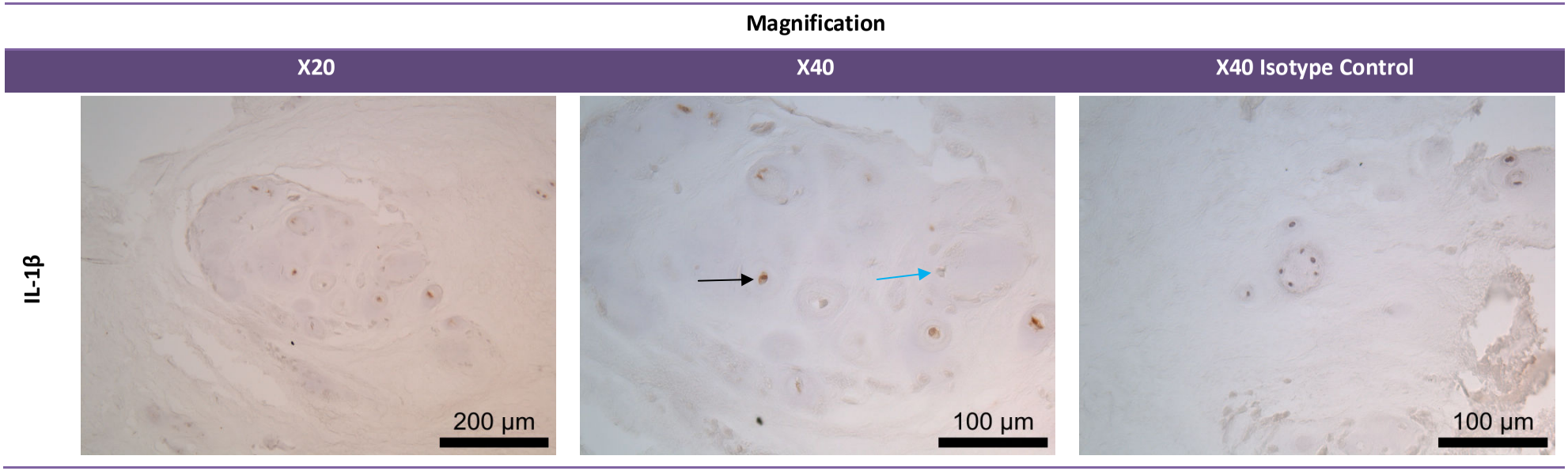


Figure 5-1 IL-1β Localisation in Human Nucleus Pulposus Tissue Sections

Production of IL-1β was localised to native NP cells. Images show; IL-1β positivity in a degenerate lesion from tissue section HD34. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

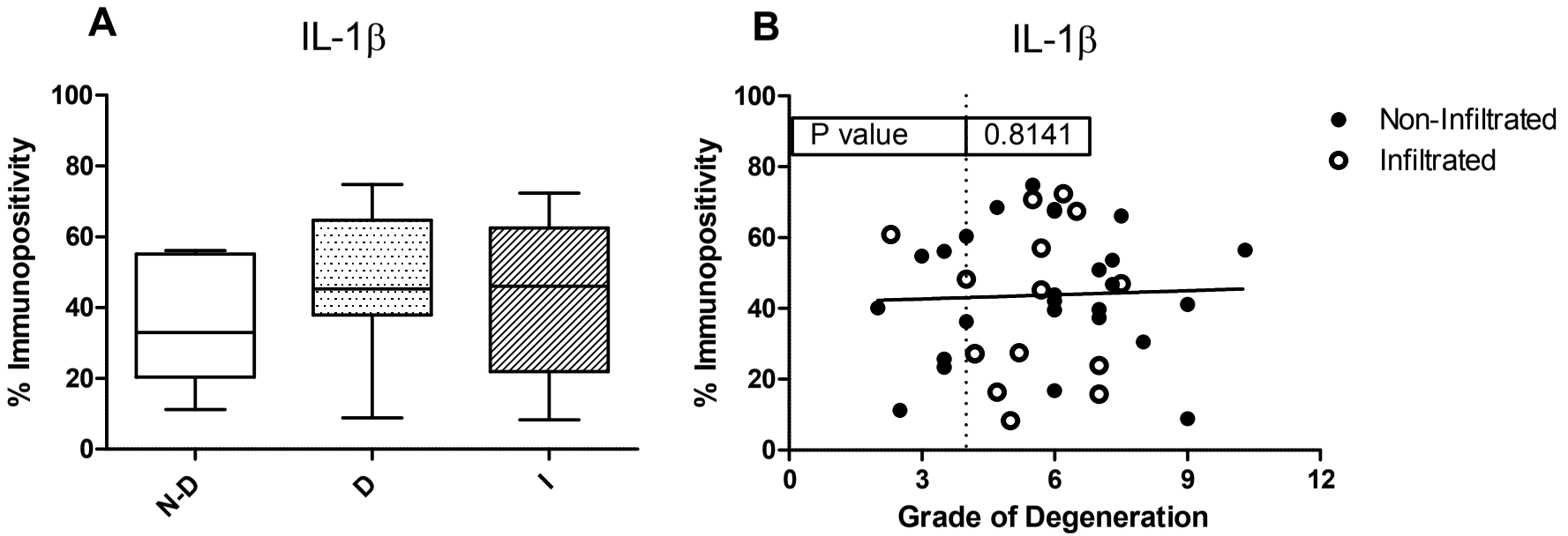


Figure 5-2 IL-1β Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 40 IVDs were investigated for IL-1β positivity, these were split between non-degenerate (N-D; n=6), degenerate (D; n=20) and infiltrated (I; n=14) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

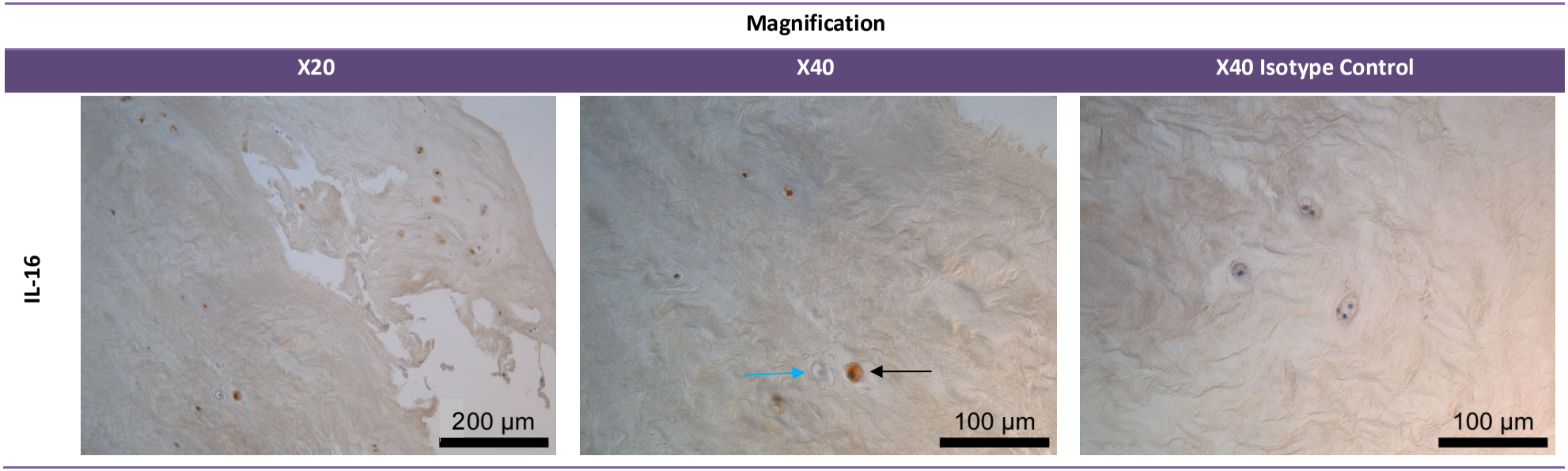


Figure 5-3 IL-16 Localisation in Human Nucleus Pulposus Tissue Sections

Production of IL-16 was localised to native NP cells. Images show; IL-16 positivity in a region of histologically normal tissue from section HD14. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

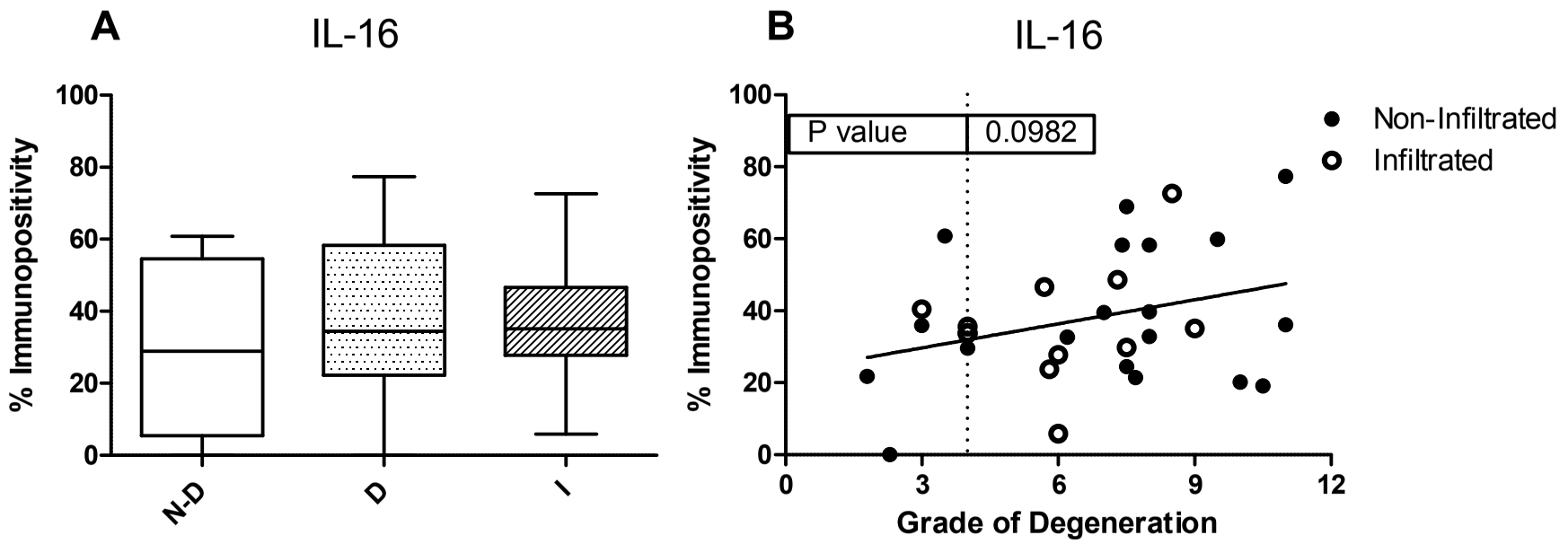


Figure 5-4 IL-16 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for IL-16 positivity, these were split between non-degenerate (N-D; n=4), degenerate (D; n=15) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

5.2.2 Chemokine Expression in Nucleus Pulposus Tissue

5.2.2.1 CCL2 Expression

CCL2 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=5) degenerate (n=14), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in all tissue sections with the exception of 1 non-degenerate. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-5).

CCL2 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-6A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 25.38%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 58.37%, and infiltrated IVDs, median 57.77%, although these differences were not significant (P=0.0526 against degenerate tissue sections, P=0.0870 against infiltrated). Regression analysis considering CCL2 immunopositivity and histologically determined grade of degeneration confirms a positive linear relationship exists between CCL2 immunopositivity and grade of degeneration in tissue sections from human IVDs (P=0.0067; Figure 5-6B).

5.2.2.2 CCL3 Expression

CCL3 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=5) degenerate (n=14), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in each tissue section with the exception of 1 from the non-degenerate study group. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-7).

CCL3 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-8A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 15.50%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 37.00%, and infiltrated IVDs, median 40.38%, compared to non-degenerate. This difference was significant against degenerate tissue sections (P=0.0186) but not infiltrated (P>0.05). Regression analysis considering CCL3 immunopositivity and histologically determined grade of degeneration confirms a positive linear relationship exists between CCL3 immunopositivity and grade of degeneration in tissue sections from human IVDs (P=0.0171; Figure 5-8B).

5.2.2.3 CCL4 Expression

CCL4 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=6) degenerate (n=13), and infiltrated (n=11) IVDs. NP cell

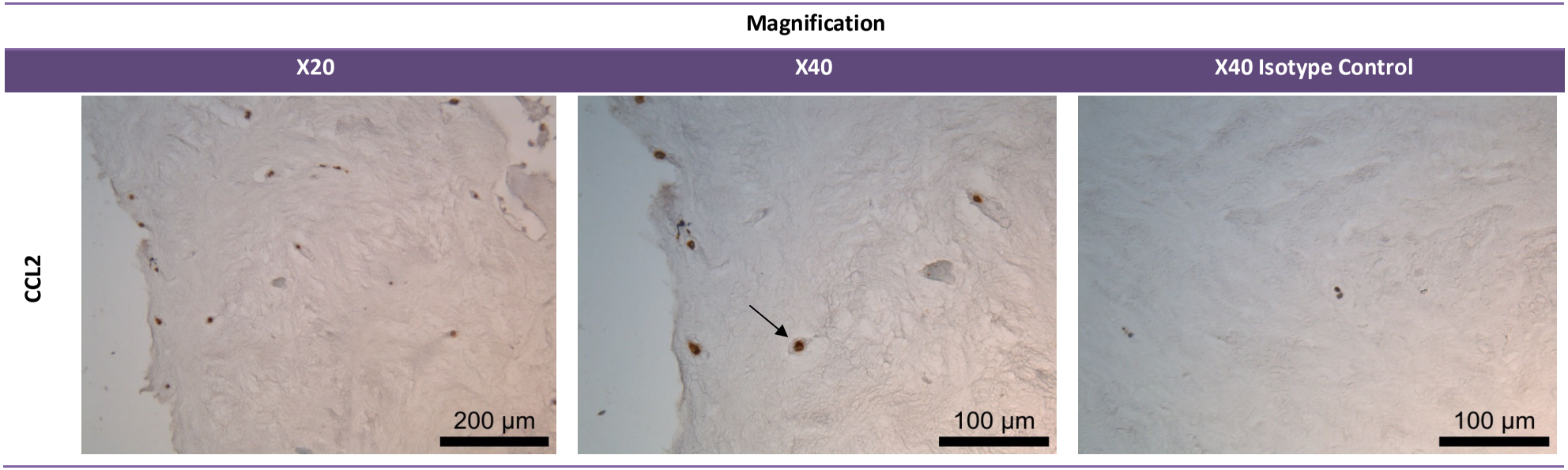


Figure 5-5 CCL2 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CCL2 was localised to native NP cells. Images show; CCL2 positivity in a mildly degenerate region of tissue section HD3. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

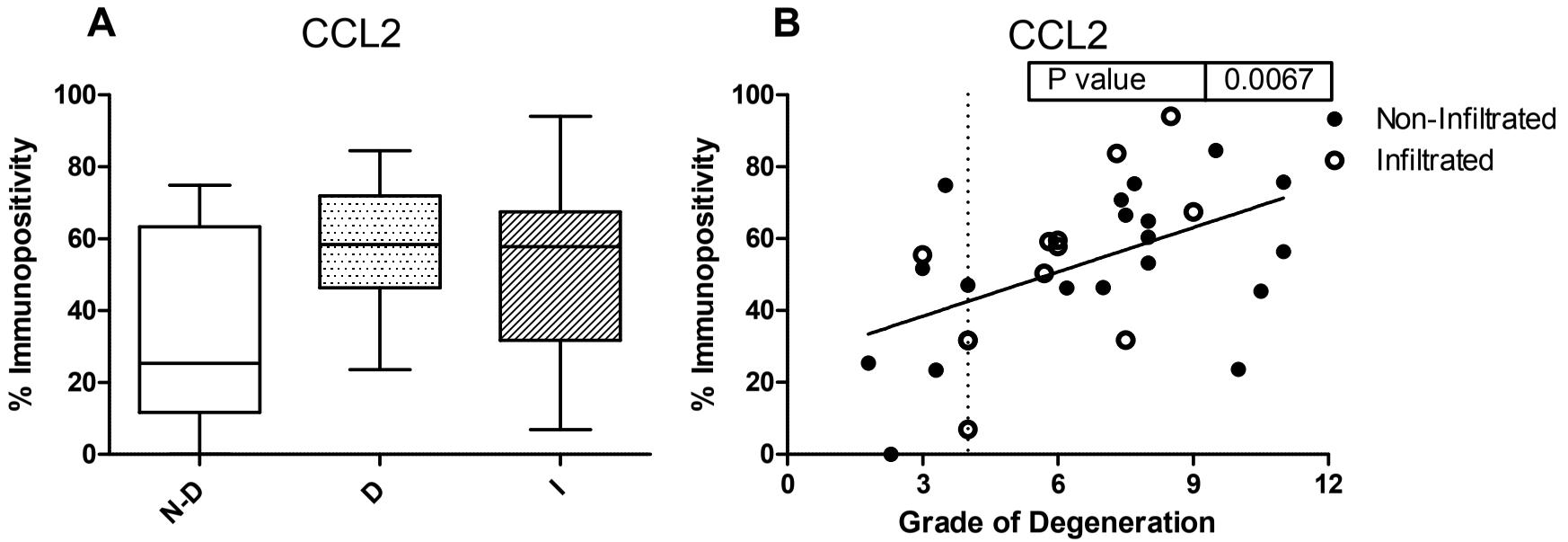


Figure 5-6 CCL2 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CCL2 positivity, these were split between non-degenerate (N-D; n=5), degenerate (D; n=14) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups (P<0.05).

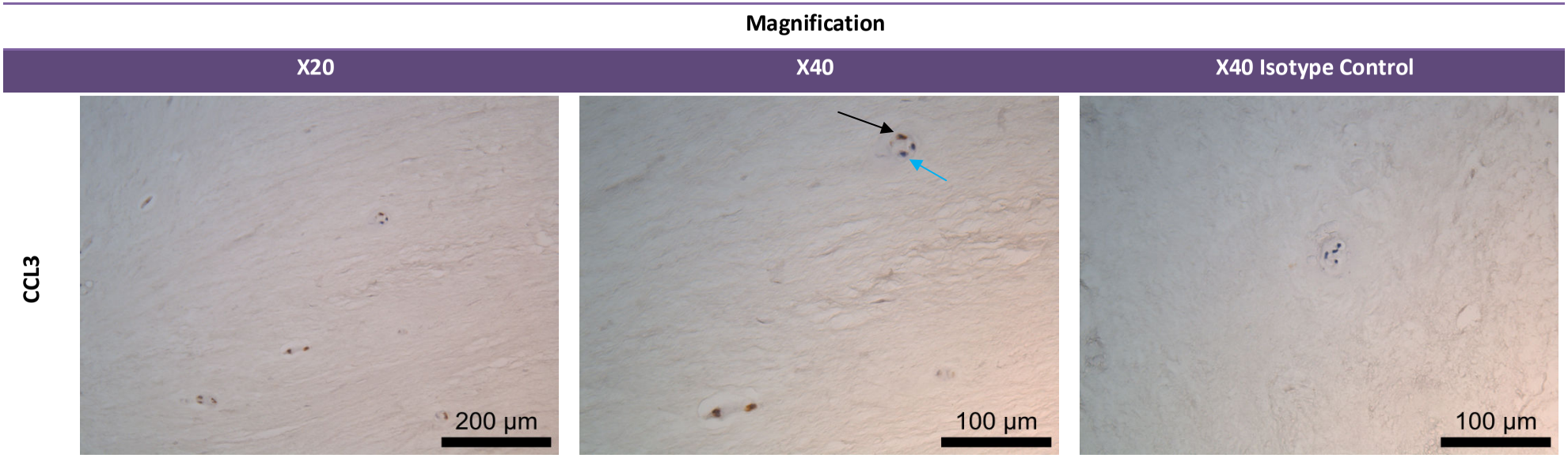


Figure 5-7 CCL3 Localisation in Human Nucleus Pulposus Tissue

Production of CCL3 was localised to native NP cells. Images show; CCL3 positivity in a region of histologically normal tissue from section HD34. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

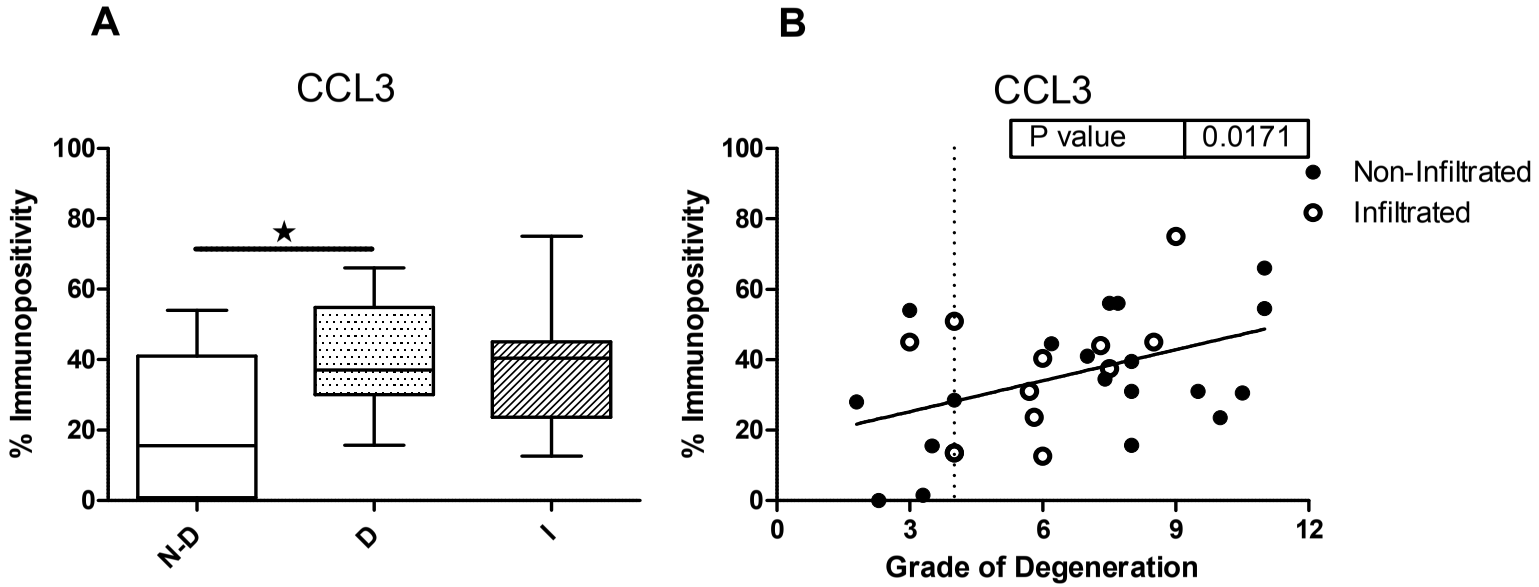


Figure 5-8 CCL3 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CCL3 positivity, these were split between non-degenerate (N-D; n=5), degenerate (D; n=14) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

immunopositivity was observed in each tissue section. In all instances staining was localised to the perinuclear region of NP cells as shown in Figure 5-9.

CCL4 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-10A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 12.39%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 23.94%, and infiltrated IVDs, median 14.90%, compared to non-degenerate. This difference was significant against degenerate tissue sections (P=0.0196), but not infiltrated (P>0.05). Regression analysis considering CCL4 immunopositivity and histologically determined grade of degeneration confirms that no linear relationship exists between CCL4 immunopositivity and grade of degeneration in tissue sections from human IVDs (P>0.05; Figure 5-10B).

5.2.2.4 CCL7 Expression

CCL7 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=5) degenerate (n=14), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in all tissue sections with the exception of 2 from the non-degenerate study group. In all instances staining was localised to the perinuclear region of NP cells as shown in Figure 5-11.

CCL7 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-12A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 5.769%. Significantly increased immunopositivity was detected in tissue sections from degenerate IVDs, median 16.06% (P=0.0288) and infiltrated IVDs, median 21.62% (P=0.0348), compared to non-degenerate. Regression analysis considering CCL7 immunopositivity and histologically determined grade of degeneration indicates a linear relationship exists between CCL7 immunopositivity and grade of degeneration in tissue sections from human IVDs although this was not significant (P=0.0600; Figure 5-12B).

5.2.2.5 CXCL8 Expression

CXCL8 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=5) degenerate (n=14), and infiltrated (n=11) IVDs. Immunopositivity was observed in all tissue sections with the exception of 2 from the degenerate study group. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-13).

CXCL8 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-14A. Immunopositivity was detected in tissue

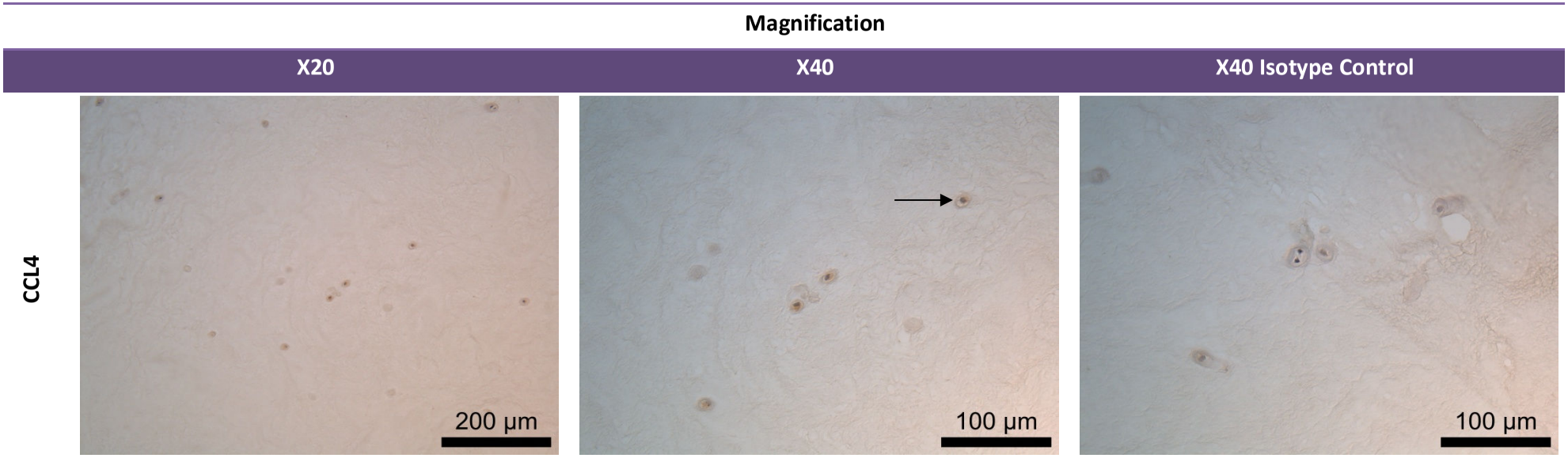


Figure 5-9 CCL4 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CCL4 was localised to native NP cells. Images show; CCL4 positivity in a region of histologically normal tissue from section HD54. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells.

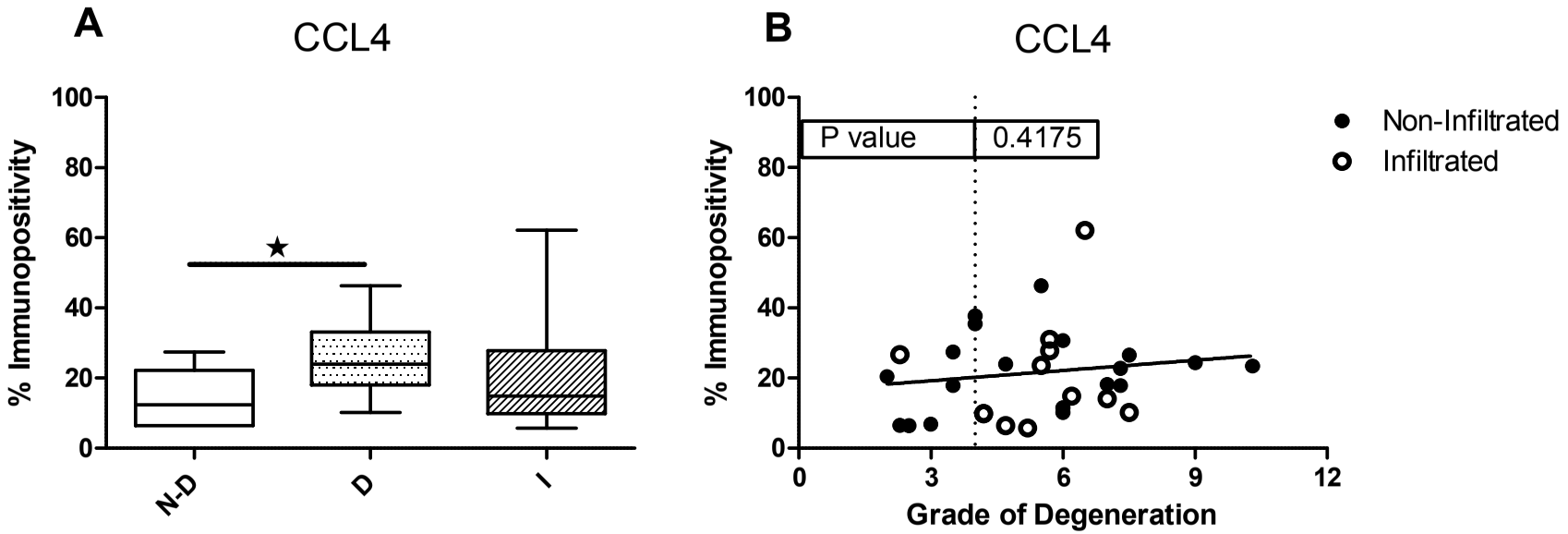


Figure 5-10 CCL4 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CCL4 positivity, these were split between non-degenerate (N-D; n=6), degenerate (D; n=13) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration. ★, significant difference in measured percentage immunopositivity between study groups (P<0.05)

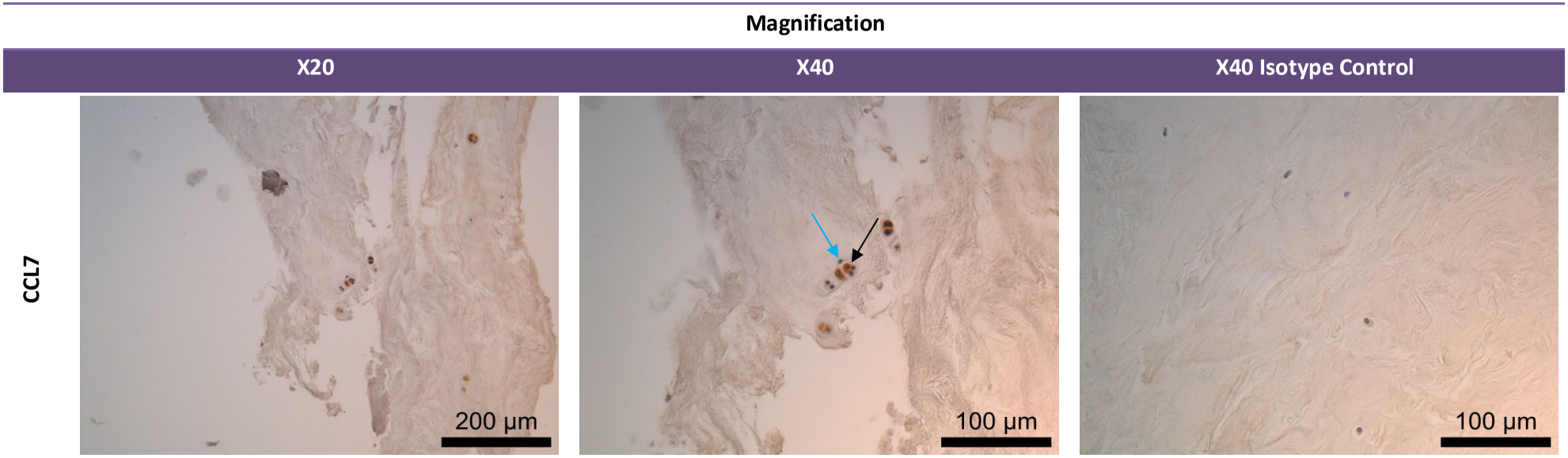


Figure 5-11 CCL7 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CCL7 was localised to native NP cells. Images show; CCL7 positivity in a moderately degenerate lesion of tissue section HD50. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

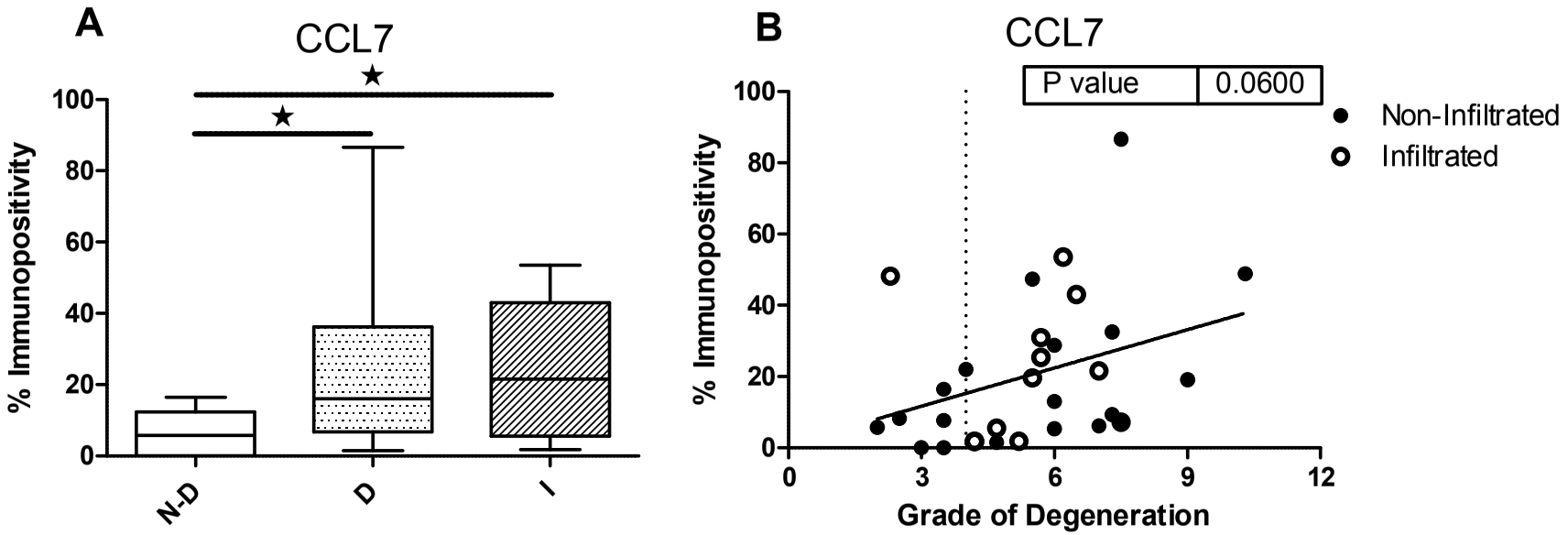


Figure 5-12 CCL7 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CCL7 positivity, these were split between non-degenerate (N-D; n=5), degenerate (D; n=14) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups (P<0.05).

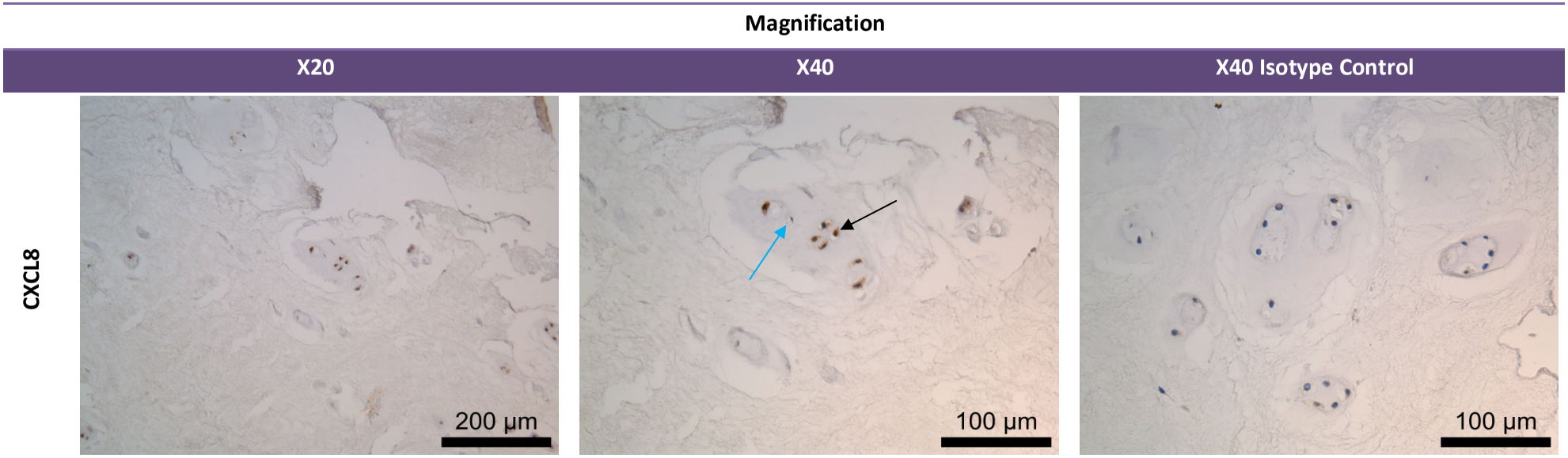


Figure 5-13 CXCL8 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CXCL8 was localised to native NP cells. Images show; CXCL8 positivity in degenerate lesion of tissue section HD36. Positivity is indicated by the presence of brown staining (black arrow). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrow).

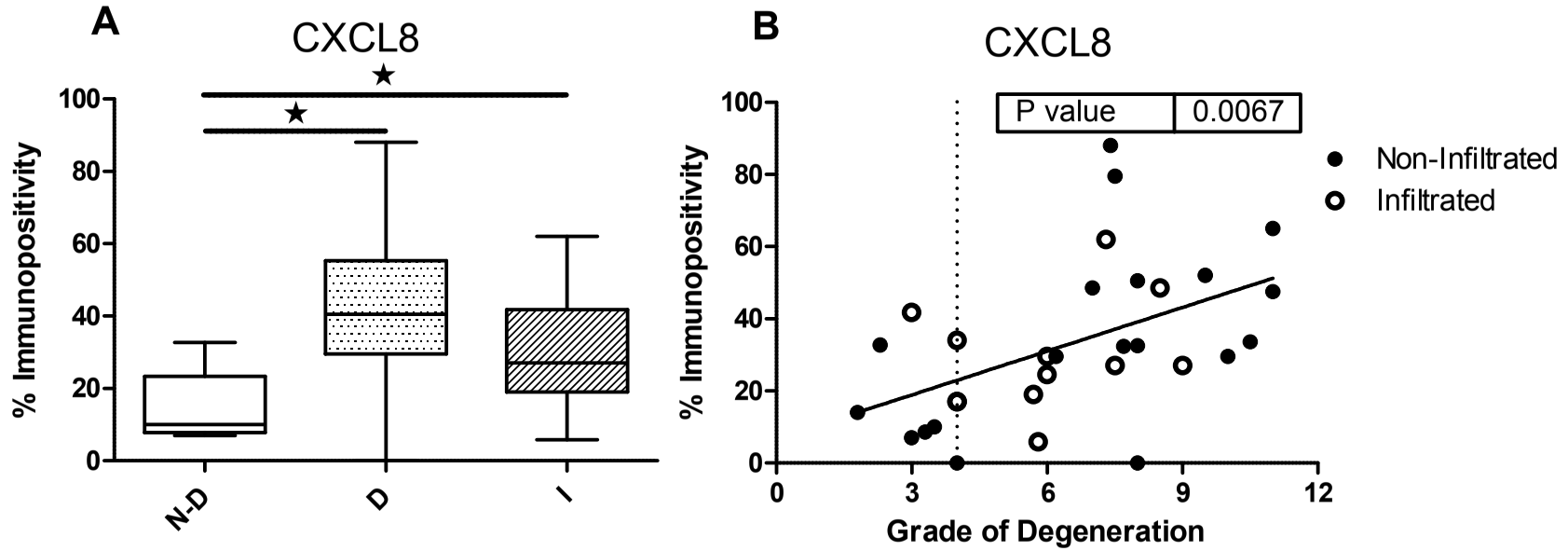


Figure 5-14 CXCL8 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CXCL8 positivity, these were split between non-degenerate (N-D; n=5), degenerate (D; n=14) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

sections from non-degenerate IVDs, median 10.00%. Significantly increased immunopositivity was detected in tissue sections from degenerate IVDs, median 40.55% (*P*=0.0287) and infiltrated IVDs, median 27.04% (*P*=0.0349), compared to non-degenerate. Regression analysis considering CXCL8 immunopositivity and histologically determined grade of degeneration confirms a positive linear relationship exists between CXCL8 immunopositivity and grade of degeneration in tissue sections from human IVDs (*P*=0.0067; Figure 5-14B).

5.2.3 Receptor Expression in NP Tissue

5.2.3.1 CD4 Expression

CD4 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=3) degenerate (n=16), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in each tissue section. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-15).

CD4 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-16A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 16.67%. Significantly increased immunopositivity was detected in tissue sections from degenerate IVDs, median 36.92% (*P*=0.0146) and infiltrated IVDs, median 54.38% (*P*=0.0098), compared to non-degenerate. Immunopositivity in infiltrated IVDs was also seen to be increased compared to degenerate (*P*=0.0228). Regression analysis considering CD4 immunopositivity in NP cells and histologically determined grade of degeneration confirms that no linear relationship exists between CD4 immunopositivity grade of degeneration in tissue sections from human IVDs (*P*>0.05; Figure 5-16B).

5.2.3.2 CCR1 Expression

CCR1 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=3) degenerate (n=16), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in each tissue section. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-17).

CCR1 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-18A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 38.91%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 46.32%, and infiltrated IVDs, median 42.92%, although these differences were not significant. Regression analysis considering CCR1 immunopositivity and histologically

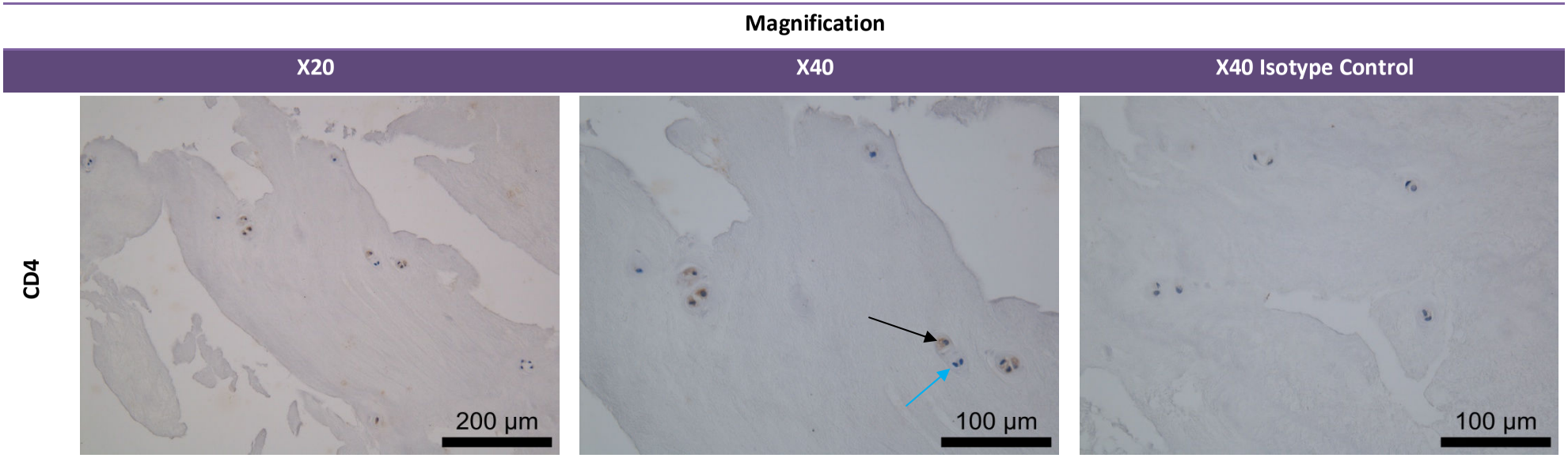


Figure 5-15 CD4 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CD4 was localised to native NP cells. Images show; CD4 positivity in a mildly degenerate lesion of tissue section HD4. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

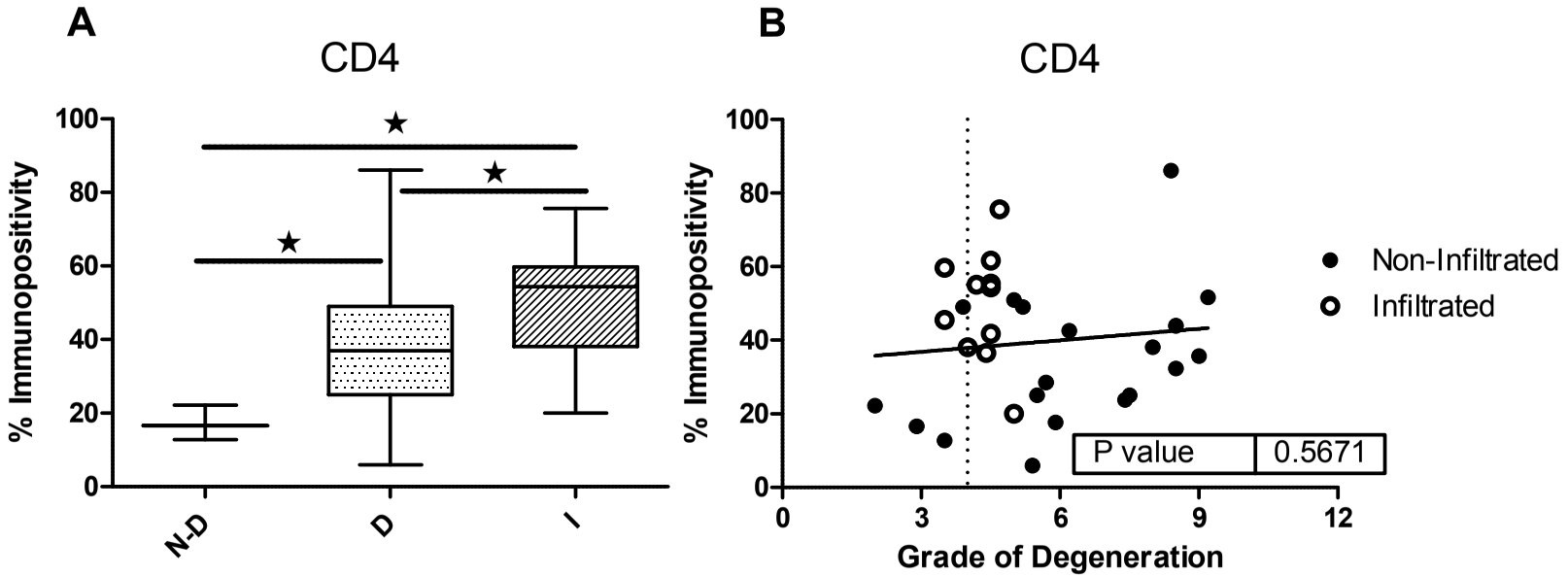


Figure 5-16 CD4 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CD4 positivity, these were split between non-degenerate (N-D; n=3), degenerate (D; n=16) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

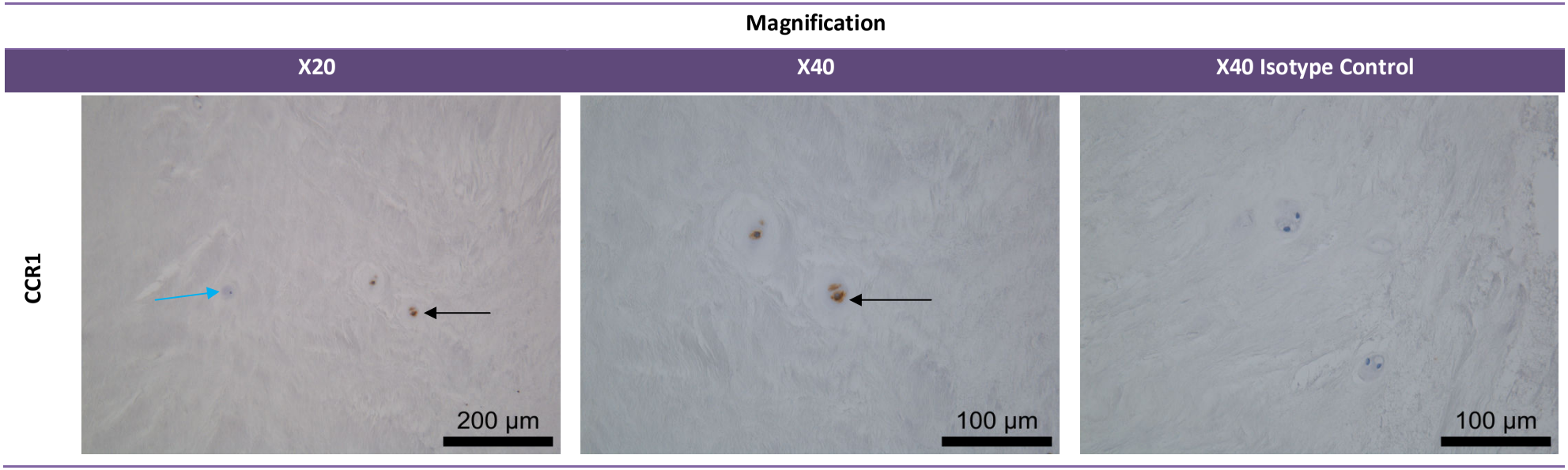


Figure 5-17 CCR1 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CCR1 was localised to native NP cells. Images show; CCR1 positivity in a histologically normal region of tissue section HD32. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

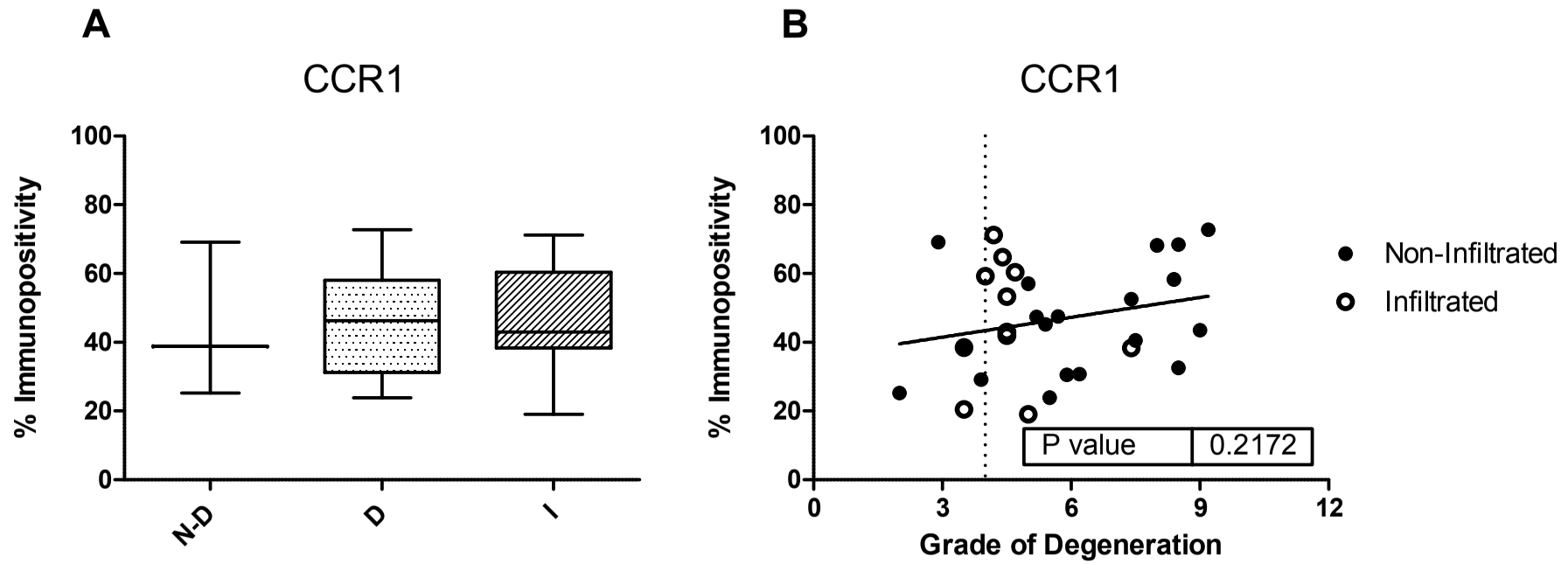


Figure 5-18 CCR1 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CCR1 positivity, these were split between non-degenerate (N-D; n=3), degenerate (D; n=16) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups (P<0.05).

determined grade of degeneration confirms that no linear relationship exists between CCR1 immunopositivity in and grade of degeneration in tissue sections from human IVDs (*P*>0.05; Figure 5-18B).

5.2.3.3 CXCR1 Expression

CXCR1 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=3) degenerate (n=15), and infiltrated (n=12) IVDs. Immunopositivity was observed in each tissue section. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-19).

CXCR1 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-20A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 86.26%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 90.05%, and infiltrated IVDs, median 89.87%, although these differences were not significant (*P*>0.05). Regression analysis considering CXCR1 immunopositivity and histologically determined grade of degeneration confirms that no linear relationship exists between CXCR1 immunopositivity and grade of degeneration in tissue sections from human IVDs (*P*>0.05; Figure 5-20B).

5.2.3.4 CXCR2 Expression

CXCR2 immunopositivity was localised to the NP cells of tissue sections from non-degenerate (n=3) degenerate (n=16), and infiltrated (n=11) IVDs. NP cell immunopositivity was observed in each tissue section. In all instances staining was localised to the perinuclear region of NP cells (Figure 5-21).

CXCR2 immunopositivity in tissue sections from non-degenerate, degenerate and infiltrated IVDs is shown in Figure 5-22A. Immunopositivity was detected in tissue sections from non-degenerate IVDs, median 55.50%. Increased immunopositivity was detected in tissue sections from degenerate IVDs, median 81.75%, and infiltrated IVDs, median 78.64%, compared to non-degenerate, although these differences were not significant (*P*>0.05). Regression analysis considering CXCR2 immunopositivity and histologically determined grade of degeneration confirms a positive linear relationship exists between CXCR2 immunopositivity and grade of degeneration in tissue sections from human IVDs (*P*=0.0371; Figure 5-22B).

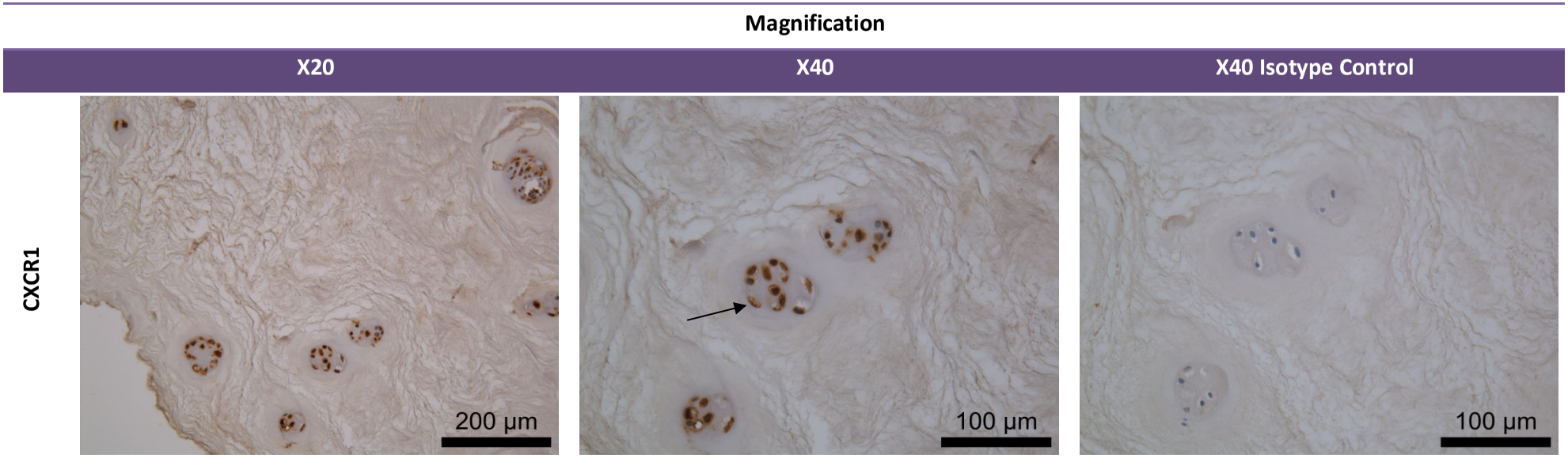


Figure 5-19 CXCR1 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CXCR1 was localised to native NP cells. Images show; CXCR1 positivity in a degenerate lesion of tissue section HD2. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

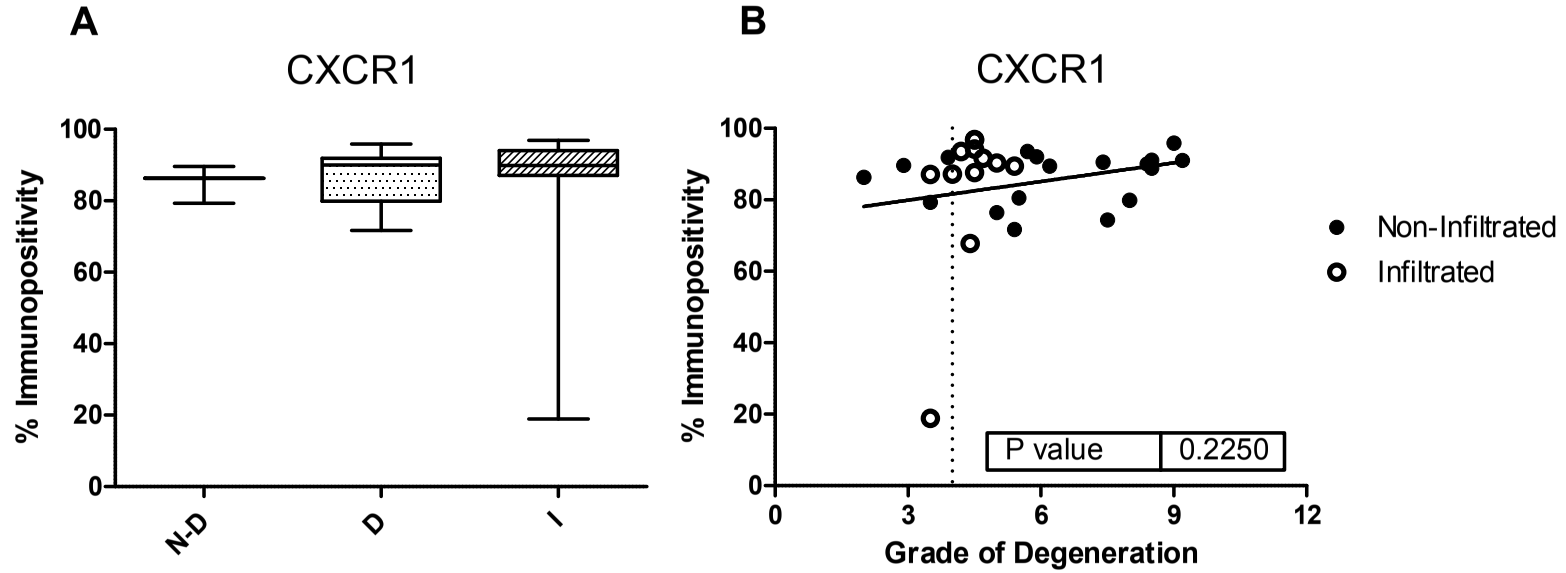


Figure 5-20 CXCR1 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CXCR1 positivity, these were split between non-degenerate (N-D; n=3), degenerate (D; n=15) and infiltrated (I; n=12) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups ($P<0.05$).

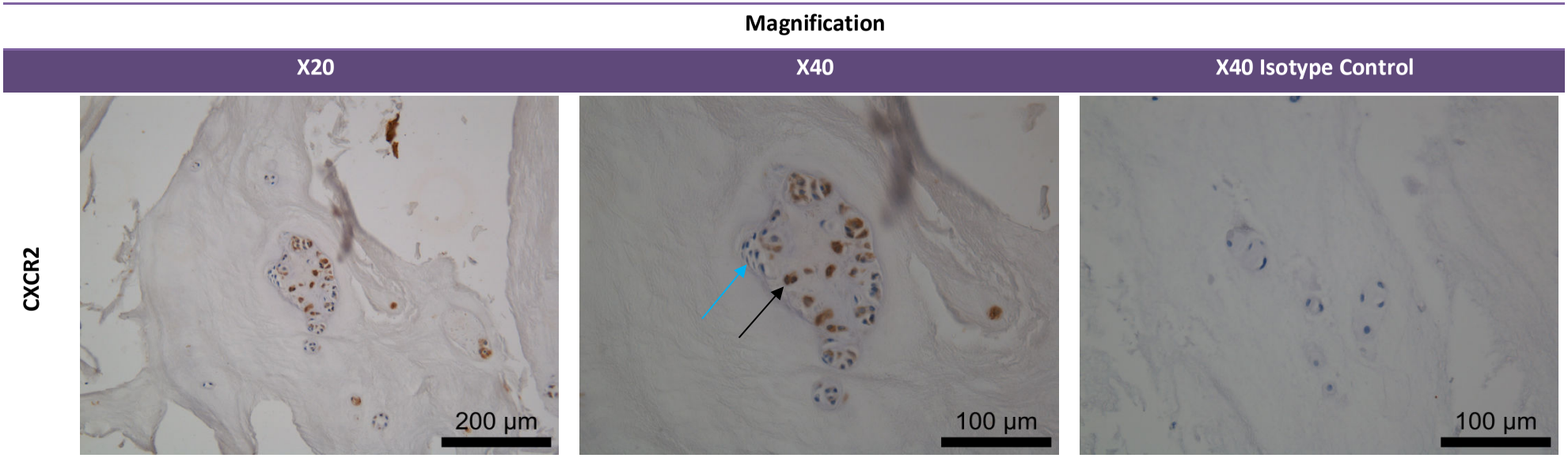


Figure 5-21 CXCR2 Localisation in Human Nucleus Pulposus Tissue Sections

Production of CXCR2 was localised to native NP cells. Images show; CXCR2 positivity in a degenerate lesion of tissue section HD52. Positivity is indicated by the presence of brown staining (black arrows). Tissue sections are counterstained with haematoxylin allowing for the identification of nuclei in negative cells (blue arrows).

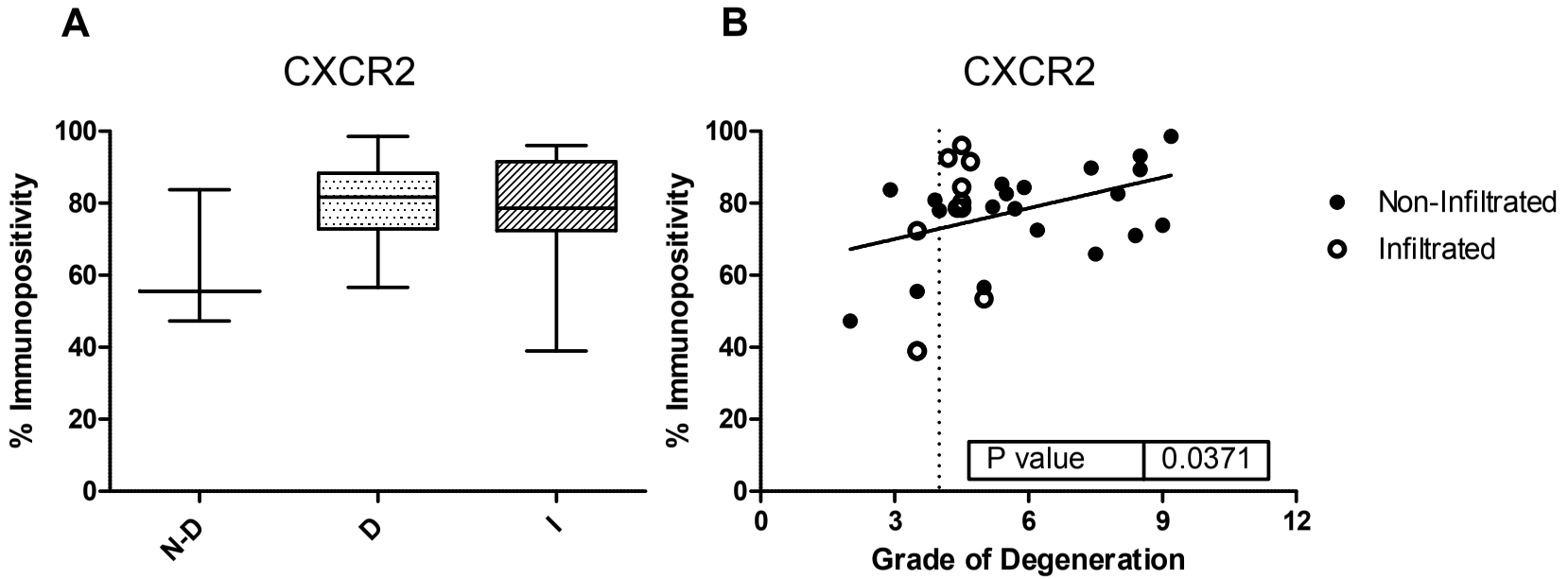


Figure 5-22 CXCR2 Immunopositivity in Nucleus Pulposus Tissue

In total, tissue sections from 35 IVDs were investigated for CXCR2 positivity, these were split between non-degenerate (N-D; n=3), degenerate (D; n=16) and infiltrated (I; n=11) study groups. For each tissue section, 200 NP cells were counted and the number of positive cells expressed as a percentage. Box and whisker plots represent the measured percentage immunopositivity across study groups. Adjacent sections from each tissue block were examined histologically to determine the grade of degeneration. Scatter plots represent regression analysis of percentage immunopositivity data against histologically determined grade of degeneration, dotted line indicates grade 4 degeneration, below which tissue samples are considered histologically normal. ★, significant difference in measured percentage immunopositivity between study groups (P<0.05).

5.2.4 Summary of Results

The results presented in this chapter are summarised in Table 5-2.

Target	Non-Degenerate	Degenerate	Infiltrated	Correlation
IL-1β	+	+ ($\uparrow_{ND}$)	+ ($\uparrow_{ND}$)	None
IL-16	+	+	+	None
CCL2	+	+ ($\uparrow_{ND}$)	+ ($\uparrow_{ND}$)	Yes (p=0.0067)
CCL3	+	+ (↑)*	+ ($\uparrow_{ND}$)	Yes (p=0.0171)
CCL4	+	+ (↑)*	+	None
CCL7	+	+ (↑)*	+ (↑)¤	Yes (p=0.0600)
CXCL8	+	+ (↑)*	+ (↑)¤	Yes (p=0.0067)
CD4	+	+ (↑)*	+ (↑)¤¥	None
CCR1	+	+	+	None
CXCR1	+	+	+	None
CXCR2	+	+ ($\uparrow_{ND}$)	+ ($\uparrow_{ND}$)	Yes (p=0.0371)

Table 5-2 Summary of Cytokine, Chemokine and Receptor Immunopositivity in Nucleus Pulposus Tissue from Non-Degenerate, Degenerate and Infiltrated Study Groups

+, protein expression detected; -, protein expression not detected; (↑), increase; (↓) decrease; ND, non-degenerate study group; D, degenerate study group; *, significant difference between degenerate and non-degenerate study groups (P<0.05); ¤, significant difference between infiltrated and non-degenerate study groups (P<0.05); ¥, significant difference between infiltrated and degenerate study groups (P<0.05).

5.3 Discussion

The objective of these investigations was to confirm protein expression of target cytokines, chemokines and receptors in human NP tissue. To localise expression to cells producing cytokines and chemokines in the IVD, and to identify differences in expression between non-degenerate, degenerate and infiltrated study groups.

Microscopic evaluation of IHC tissue sections confirmed protein expression of all targets investigated and localised this expression to the native NP cells of the IVD. Comparative analysis of percentage immunopositivity data revealed a trend of up-regulated cytokine and chemokine expression in both degenerate and infiltrated study groups when compared to non-degenerate. For certain targets, receptor expression was also up-regulated in both degenerate and infiltrated samples.

It is worth noting at this point that protein expression levels represented by immunopositivity are only semi-quantitative. The immunopositivity value represents the frequency that expression was observed in native NP cells, rather than the amount of protein that was being produced. As such, it might be expected that the amount of protein, particularly for the chemokine targets, would be greater in infiltrated samples as this may drive the chemotaxis of leukocytes into the tissue and/or capillary in growth which facilitates increased infiltration (Gerard & Rollins, 2001; Thelen, 2001; Gangur *et al.*, 2002; Gerard, 2005; Wolf *et al.*, 2008; Kiefer & Siekmann, 2011). Indeed, this may be the case – even though few significant increases in immunopositivity were observed here between non-infiltrated degenerate samples and infiltrated, the amount of protein produced per positive cell may be greater in the infiltrated samples and therefore account for the leukocyte immigration.

5.3.1 Cytokines

IL-1β expression was localised to NP cells, as has been described previously (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d). Comparative analysis between study groups indicates increased expression in degenerate and infiltrated samples however, these findings were not significant. This trend mirrors that observed for IL-1β gene expression across the same study groups (see Chapter 4, section 4.2.1.1) with the exception that gene expression in infiltrated samples was significantly increased compared to non-degenerate whereas here, the observed increase in protein expression was not significant. However, immunopositivity quantification in this investigation was limited to that by native NP cells. Previous gene expression data included expression by infiltrating cells and this may explain the greater increase observed in gene expression in the infiltrated study group.

Previously, significant up-regulation of IL-1β protein expression has been described in both degenerate and prolapsed IVDs compared to normal (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d). Whilst a trend of up-regulation in degeneration was observed here our data is not entirely in agreement with previously published reports and this may be related to an altered IL-1β expression profile in prolapsed tissue.

IL-16 expression was confirmed in the human IVD and expression localised to native NP cells. The IL-16 receptor, CD4 (Cruikshank *et al.*, 2000; Bernstein *et al.*, 2006; Liu *et al.*, 2007; Fenster *et al.*, 2010), was also localised to native NP cells. Neither target has been reported previously in the IVD, and similarly there are no reports of expression by chondrocytes. Hence, we report a novel cytokine and receptor system potentially active through autocrine or paracrine signalling in the IVD. IL-16 protein expression was equivalent across study groups, a trend mirrored in previous gene expression studies (see Chapter 4, section 4.2.1.1). However, receptor CD4 protein expression was increased in degenerate and infiltrated study groups compared to non-degenerate. This may indicate increased sensitivity to IL-16 signalling by NP cells in degeneration. Interestingly the increase in protein expression observed between degenerate and infiltrated study groups was also significant, indicating that factors regulating NP cell CD4 expression are further increased within the IVD by the presence of infiltrating cells, or that NP cell CD4 expression enhances leukocyte infiltration. The observed protein expression pattern for CD4 is altered from that observed for CD4 gene expression where relative gene expression levels were equivalent between study groups, although detection frequency was increased in degenerate samples compared to non-degenerate (see Chapter 4, section 4.2.1.1). This would indicate translational regulatory mechanisms modulate the observed increases in CD4 protein expression, particularly in infiltrated samples. Of note, two other studies have investigated CD4 expression in the human IVD, by IHC for the purpose of identifying CD4+ infiltrating T lymphocytes (Kawaguchi *et al.*, 2001; Shamji *et al.*, 2010). Kawaguchi *et al.* (2001) reported that the human IVD is CD4 negative however, primary antibody specificity and reaction conditions were not confirmed by use of positive control tissue and this may be one explanation for the conflict with data presented here. Shamji *et al*. (2010) reported that <20% of cells stained positive for CD4 by IHC in tissue sections from degenerate and prolapsed IVDs.

To return to a point of discussion un-resolved in Chapter 3, data generated in this investigation confirms the CD4 expression identified previously in gene expression

analysis, is attributable to NP cell expression and not derived entirely from T lymphocyte infiltration. Indeed, although CD4 expression is classically associated with the T lymphocyte (Berman *et al.*, 1985), the discovery that IL-16 requires cell surface expression of CD4 for induction of all its known biological activities (Cruikshank *et al.*, 1991; Ryan *et al.*, 1996) has led to the identification of other CD4 positive target cells of IL-16. These include other immune cells such as monocytes (Cruikshank *et al.*, 1987), eosinophils (Rand *et al.*, 1991; Wan *et al.*, 1995; Lim *et al.*, 1996) and dendritic cells (Kaser *et al.*, 1999), some neuronal cells are also identified as CD4 expressing (Kurschner & Yuzaki, 1999; Fenster *et al.*, 2007). Of the known biological activities of IL-16, most are associated with cell proliferation (Cruikshank *et al.*, 1987; Parada *et al.*, 1998) and differentiation (Szabo *et al.*, 1998) although IL-16 is known to possess potent chemo-attractant properties to CD4 positive cells (Berman *et al.*, 1985) and modulates the adhesion to ECM proteins by eosinophils (Wan *et al.*, 1995).

5.3.2 Chemokines

Expression of the C-C chemokines; CCL2, CCL3, CCL4 and CCL7, and receptor CCR1 was localised to NP cells of the IVD. NP cell CCL2 production has been identified previously by IHC in the prolapsed IVD (Ohba *et al.*, 2008). Data presented here indicated that CCL2 production is increased in degenerate and infiltrated tissue samples compared to non-degenerate, and that protein production is increased in line with increasing severity of degenerative tissue changes. Increased CCL2 protein production by prolapsed IVDs has been described previously where CCL2 concentration in conditioned media from tissue culture explants was interrogated for CCL2 presence by ELISA (Burke *et al.*, 2002). This same study reported CCL2 detection in culture supernatants from 40% of control tissue explants investigated (Burke *et al.*, 2002). Our study identified immunopositivity in 4 of the 5 (80%) non-degenerate samples investigated and this increase may be related to the use of prolapsed non-degenerate samples in this study. The differential CCL2 protein expression profile observed here between non-degenerate and degenerate study groups revealed the opposite trend of up-regulation in degeneration when compared to our previous gene expression investigation that indicated decreased expression in degeneration (see Chapter 4, section 4.2.2.1), and may indicate that translational modulation results in the observed increase in protein production. The differential CCL2 protein expression profile observed here between non-degenerate and infiltrated study groups is similar to that observed previously in gene expression analysis (see Chapter 4,

section 4.2.2.1), in both cases a trend of increased expression in infiltrated samples was observed although this was not significant in either case.

This is the first report of CCL3, CCL4 and CCL7 protein production by NP cells. Production of CCL3 and CCL4 has been investigated previously by IHC in articular cartilage from normal and OA tissue donors where the 3 normal tissue samples and 5 OA tissue samples investigated were negative for CCL3 and CCL4 immunostaining (Yuan *et al.*, 2001). This same study also reported that cultured primary human articular chondrocytes, derived from normal and OA tissue donors were CCL3 and CCL4 negative when assessed by immunocytochemistry, but that expression of both chemokines was induced following the application of pro-inflammatory stimulus (IL-1 treatment) (Yuan *et al.*, 2001) indicating that chondrocytes do have the capacity to produce these chemokines. CCL7 protein production has not been investigated previously in NP cells or chondrocytes however, gene expression has been reported in prolapsed IVDs (Kawaguchi *et al.*, 2002) and our earlier gene expression investigations demonstrated NP cell gene expression (see Chapter 3, section 3.2.2.1 and Chapter 4, section 4.2.2.1).

Comparative analysis between non-degenerate and degenerate study groups indicates CCL3, CCL4 and CCL7 production is increased in degeneration. These trends were not observed previously in our gene expression investigation where relative mRNA transcription levels were equivalent between these same study groups (see Chapter 4, section 4.2.2.1). Production of CCL3 and CCL7 but not CCL4, was seen to increase in line with increasing severity of degenerative tissue changes.

Differential protein expression profiles were observed for CCL3 and CCL7 but not CCL4, between non-degenerate and infiltrated study groups. For CCL3, our previous gene expression analysis identified an increase in the frequency of CCL3 gene expression in infiltrated samples compared to non-degenerate (see Chapter 4, section 4.2.2.1) and this was confirmed here at the level of protein production by NP cells. For CCL4 our previous gene expression analysis indicated that relative mRNA expression levels were equivalent between non-degenerate and infiltrated study groups (see Chapter 4, section 4.2.2.1), a trend mirrored here at the level of protein production. For CCL7, our previous gene expression analysis indicated that relative mRNA transcription levels were equivalent between study groups (see Chapter 4, section 4.2.2.1), whilst here protein production by NP cells was increased in infiltrated samples compared to non-degenerate samples.

The differences highlighted between the protein production and gene expression profiles of CCL3, CCL4 and CCL7 in non-degenerate and degenerate samples, and CCL7 in non-degenerate and infiltrated samples may have arisen from differences in stability between mRNA and protein molecules. The half-life of mRNA is often short while protein molecules are more stable and remain detectable over greater time periods (Villiger *et al.*, 1992). It is also possible that translational regulatory mechanisms rather than transcriptional, modulate C-C chemokine expression by NP cells in these situations.

CCR1 expression was confirmed to native NP cells indicating that NP cells are both the producers and targets of C-C chemokines. This is the first report of chemokine receptor expression by NP cells and opens the possibility of autocrine and paracrine signalling mechanisms for chemokines in the IVD. CCR1 expression was equivalent across study groups with approximately 40% immunopositivity of cells in all sections investigated. This finding is remarkably similar to chondrocyte immunopositivity for CCR1 in articular cartilage where 37% (median value) immunopositivity (assessed by IHC) is reported in cartilage from normal tissue donors with no significant alteration in expression in OA tissue donors (Silvestri *et al.*, 2003). The CCR1 protein expression pattern here mirrors data generated in previous gene expression investigations between the same study groups (see Chapter 4, section 4.2.2.1).

Expression of CXCL8 and receptors CXCR1 and CXCR2 were localised to the NP cells of the IVD. CXCL8 has been identified previously as a product of NP tissue by interrogation of conditioned media from explant cultures (Burke *et al.*, 2002). This study reported detection of CXCL8 in conditioned media from 20% of control tissue explants investigated (Burke *et al.*, 2002). In these studies CXCL8 immunopositivity was detected in 5 of 5 (100%) non-degenerate samples investigated and this difference may be related to the use of prolapsed tissue in this study since increased frequency of detection and increased expression levels of CXCL8 were observed in conditioned media for prolapsed NP tissue explants (Burke *et al.*, 2002). Although, in this study 3 of the 5 non-degenerate tissue sections investigated were from non-prolapsed, non-degenerate post-mortem IVDs, all of which were positive for CXCL8 production. The increased detection of CXCL8 reported in this study may therefore be due in part to differences in detection techniques. Here, production and localisation by IHC identified CXCL8 production in all non-degenerate tissue samples. Previously, Burke *et al.*, (2002) detected CXCL8 in conditioned media from non-degenerate NP tissue explants,

although in that study the actual parameter measured is protein release from the explant culture system and this may explain the differences reported.

Comparative analysis across study groups indicates CXCL8 expression is increased in both degenerate and infiltrated samples compared to non-degenerate, a trend that was observed previously in CXCL8 gene expression analysis (see Chapter 4, section 4.2.2.2). Protein expression analysis indicates CXCL8 expression increases in line with increasing severity of degenerative tissue changes within the NP.

This is the first report of C-X-C chemokine receptor protein expression by NP cells although expression of CXCR1 and CXCR2 has been reported previously by chondrocytes in articular cartilage (Silvestri *et al.*, 2003). This study reported CXCR1 and CXCR2 immunopositivity in articular cartilage from normal tissue donors at 45% with no significant alteration in expression pattern in tissue from OA tissue donors (Silvestri *et al.*, 2003). Here, measured immunopositivity in non-degenerate NP tissue was 86% for CXCR1 and 55% for CXCR2, this increased expression pattern from that in articular cartilage may be a tissue specific difference.

Comparative analysis indicates that CXCR1 protein production is equivalent across non-degenerate, degenerate and infiltrated study groups, a trend observed previously in gene expression analysis (see Chapter 4, section 4.2.2.2). Comparative analysis indicates CXCR2 protein production is increased in degenerate and infiltrated samples compared to non-degenerate, a trend observed previously in gene expression analysis (see Chapter 4, section 4.2.2.2). CXCR2 protein expression analysis also indicates that production increases in line with increasing severity of degenerative tissue changes. These investigations indicate C-X-C receptor expression is increased in NP tissue compared to cartilage (Silvestri *et al.*, 2003) and indicates the potential of NP cells to respond to produced C-X-C chemokines.

5.3.3 Summary

Data presented in this chapter confirms protein expression of cytokines, chemokines and receptors within the human IVD. Further, cytokine and chemokine expression is increased in the degenerate and prolapsed IVD, particularly for the targets; IL-β, CCL2, CCL3, CCL4 and CXCL8.

6 The Effects of Cytokine and Chemokine Treatment on Cytokine, Chemokine, Extracellular Matrix Component and Extracellular Matrix Degrading Enzyme Gene Expression in Primary Human Nucleus Pulposus Cells *In Vitro*

6.1 Introduction

This chapter describes investigations to determine the *in vitro* effects of cytokines; IL-1β and IL-16, and chemokines; CCL2, CCL3, CCL7 and CXCL8, on gene expression by primary NP cell cultures. Real-time qPCR was used to determine the effect of cytokine or chemokine treatment on mRNA expression of cytokines, chemokines and ECM genes.

6.1.1 Study Design

6.1.1.1 Samples

Primary NP cultures derived from 13 tissue samples; HD2, HD4, HD6, HD8, HD9, HD23, HD54, HD56, HD75, HD79, HD85, HD86 and HD89 were used in these studies. Full details of samples used in this study can be found in Appendix 1.

6.1.1.2 Selection of Culture System

Initial investigations into the *in vitro* effects of IL-1β, CCL2 and CXCL8 were performed using monolayer cultures. Comparison of the basal cytokine and chemokine expression profile of monolayer cultures with the expression profile of cells isolated directly from NP tissue revealed alterations in expression patterns, possibly associated with a phenotype 'shift' and dedifferentiation. Basal cytokine and chemokine expression profiles following alginate culture were seen to resemble more closely the expression profile of cells isolated directly from tissue (Figure 6-1) and all subsequent investigations were performed on alginate cultures.

6.1.1.3 Treatments

The cytokines; IL-1β and IL-16, and the chemokines; CCL2, CCL3, CCL7 and CXCL8 were selected as culture treatments as discussed in Chapter 3, section 3.3.3. All treatments were 48 hours duration, performed on cultures derived from at least 3 different tissue samples, and performed in triplicate on cultures from each sample. Monolayer cultures were subjected to treatment with IL-1β, CCL2 or CXCL8 as described in Chapter 2, section 2.4.3. Alginate cultures were subjected to treatment with IL-1β, IL-16, CCL3 or CCL7 as described in Chapter 2, section 2.4.6.

6.1.1.4 Target Genes

Target cytokines; IL-1β and IL-16, and target chemokines; CCL2, CCL3, CCL7 and CXCL8 were selected for gene expression analysis to determine the effects of treatment on cytokine and chemokine gene expression. Two key ECM components and 3 major ECM degrading enzymes were also selected for gene expression analysis to determine the effects of treatment on ECM gene expression:

- Aggrecan is a major proteoglycan constituent of NP ECM (Stevens *et al.*, 1979). Its hydrophilic nature confers some of the structural capacity of the

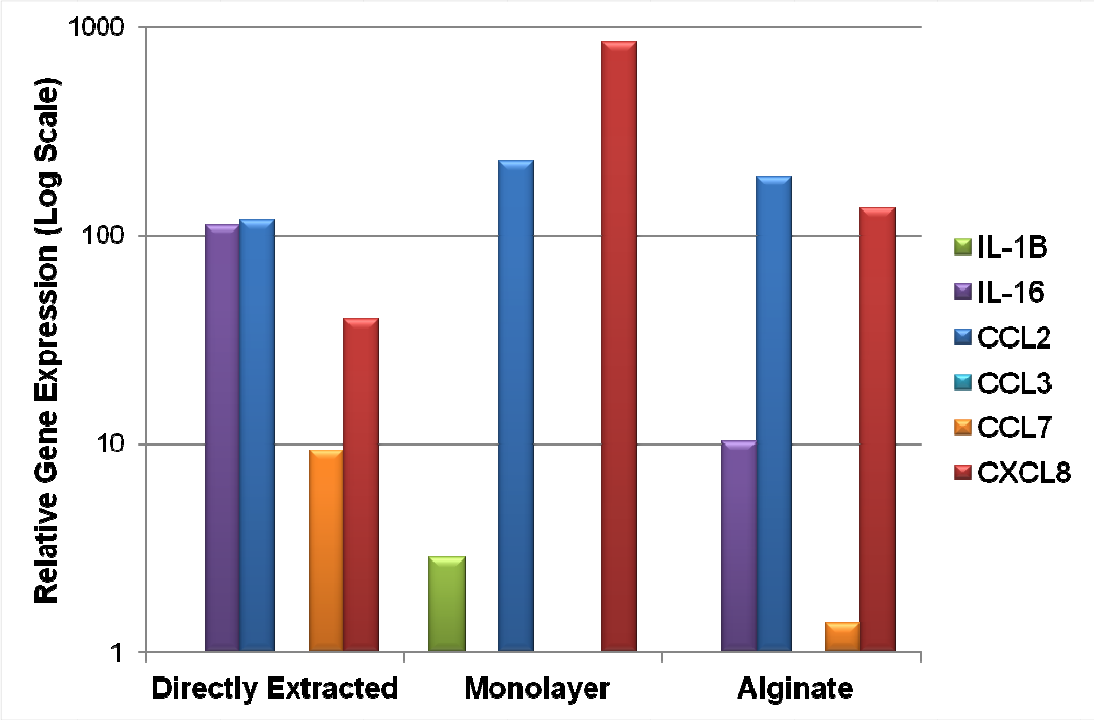


Figure 6-1 Comparison of Basal Cytokine and Chemokine Gene Expression in Directly Extracted Nucleus Pulposus Cells and those Cultured in Monolayer and Alginate Beads

Data shown relates to NP cells derived from sample HD9. Real-time qPCR analysis of cDNA derived from cells isolated directly from NP tissue revealed a profile characterised by expression of IL-16, CCL2, CCL7 and CXCL8. Following expansion in monolayer expression of CCL2 and CXCL8 was maintained but IL-16 and CCL7 was lost and IL-1β expression had been induced. Monolayer cultures were seeded to alginate and 14 days post encapsulation the expression profile was again characterised by expression of IL-16, CCL2, CCL7 and CXCL8, the IL-1β expression induced in monolayer had been lost. CCL3 expression was not observed in sample HD9. Relative gene expression was calculated by comparative quantification method (see Chapter 2, section 2.5.6) normalised to internal reference gene expression (GAPDH and 18S).

IVD (Roughley *et al.*, 2006) and decreased proteoglycan content is associated with the pathogenesis of IVD degeneration (Urban & McMullin, 1988).

- Type II collagen expression is a phenotypic marker of NP cells (Chelberg *et al.*, 1995) and type II is the prevalent collagen of NP ECM (Taylor *et al.*, 1992). Alteration in the proportions of different collagen fibres is a feature of IVD degeneration (Antoniou *et al.*, 1996a).
- MMP-3 (stromelysin) has broad substrate specificity against proteoglycan core proteins (Nemoto *et al.*, 1997). Strong immunopositivity correlations have been made between expression and the NP fissures that are common features of IVD degeneration (Weiler *et al.*, 2002).
- MMP-13 (collagenase) has proteolytic activity against collagens (I, II and III), gelatin and aggrecan – all key NP ECM constituents (Roberts *et al.*, 2000).
- ADAMTS-4 has specific aggrecanase activity and is produced within the IVD (Pockert *et al.*, 2009).

6.1.2 Methods

Full details of methods used in this study are given in Chapter 2, sections:

- 2.1 Tissue Sample Processing
- 2.4 Cell culture
- 2.5 Quantitative Polymerase Chain Reaction

6.1.3 Specific Objectives

This chapter aims to address the hypothesis that:

Regulatory inter-relationships exist between the cytokines and chemokines of the IVD and these cytokines and chemokines modulate anabolic and catabolic metabolism.

The specific objectives are:

- To determine *in vitro* the regulatory potential of IL-1β, IL-16, CCL2, CCL3, CCL7 and CXCL8 on cytokine and chemokine gene expression by primary NP cultures
- To determine *in vitro* the regulatory potential of IL-1β, IL-16, CCL2, CCL3, CCL7 and CXCL8 on Aggrecan, type II collagen, MMP-3, MMP-13 and ADAMTS-4 gene expression by primary NP cultures
- To select potentially regulatory cytokines and chemokines for further *in vitro* investigations into cytokine and chemokine protein expression in primary NP cultures

6.2 Results

6.2.1 Cytokine and Chemokine Gene Expression in Monolayer Cultures

6.2.1.1 IL-1β mRNA Expression

Constitutive IL-1β mRNA expression was observed under basal conditions in monolayer cultured NP cells derived from the 5 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by up-regulating IL-1β mRNA expression (Figure 6-2). Up-regulation of IL-1β mRNA was observed separately in cells derived from all 5 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in an 8.62 fold increase (P=0.0097), treatment at 10ng/mL resulted in a 16.67 fold increase (P=0.0019) and treatment at 100ng/mL resulted in a 12.73 fold increase (P=0.003) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No alteration in IL-1β mRNA expression was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10 or 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-2).

6.2.1.2 CCL2 mRNA Expression

Constitutive CCL2 mRNA expression was observed under basal conditions in monolayer cultured NP cells derived from all 5 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by up-regulating CCL2 mRNA expression (Figure 6-3). Up-regulation of CCL2 mRNA expression was observed separately in cells derived from all 5 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in a 5.33 fold increase (P=0.0003), treatment at 10ng/mL resulted in a 9.72 fold increase (P<0.0001) and treatment at 100ng/mL resulted in a 5.32 fold increase (P<0.0001) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No alteration in CCL2 mRNA expression was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10 or 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-3).

6.2.1.3 CXCL8 mRNA Expression

Constitutive CXCL8 mRNA expression was observed under basal conditions in monolayer cultured NP cells derived from all 5 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by up-regulating CXCL8 mRNA expression (Figure 6-4). Up-regulation of CXCL8 mRNA expression was observed separately in cells derived from all 5 IVDs investigated (data not

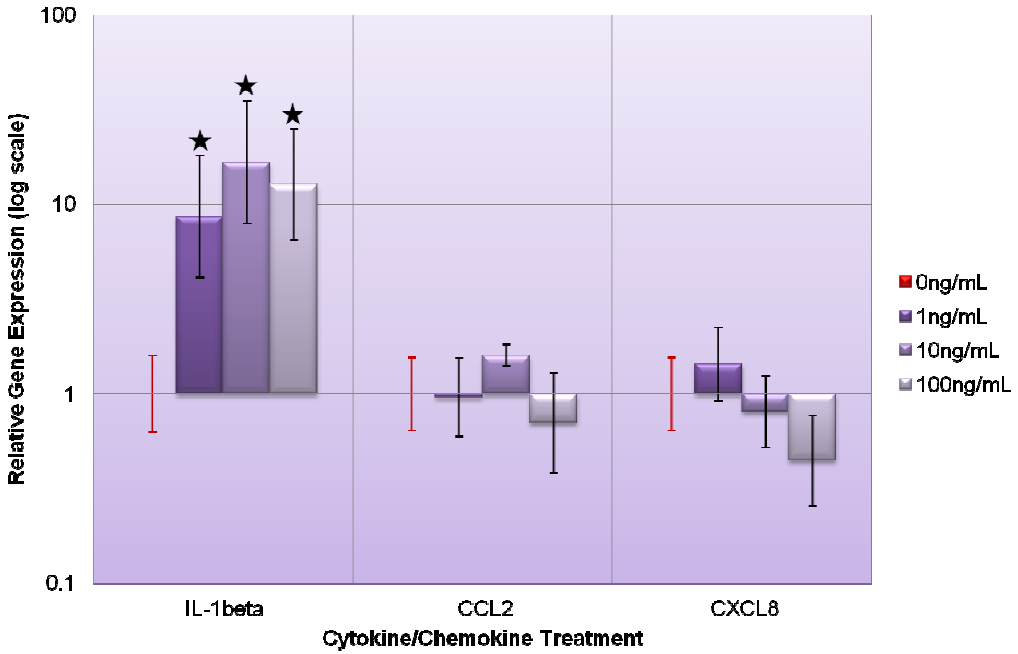


Figure 6-2 IL-1β mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each IL-1β treatment concentration was performed in triplicate on cultures derived from five different NP tissue samples (see **Table 2-8** for details of samples used). Each CCL2 and CXCL8 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). IL-1β mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

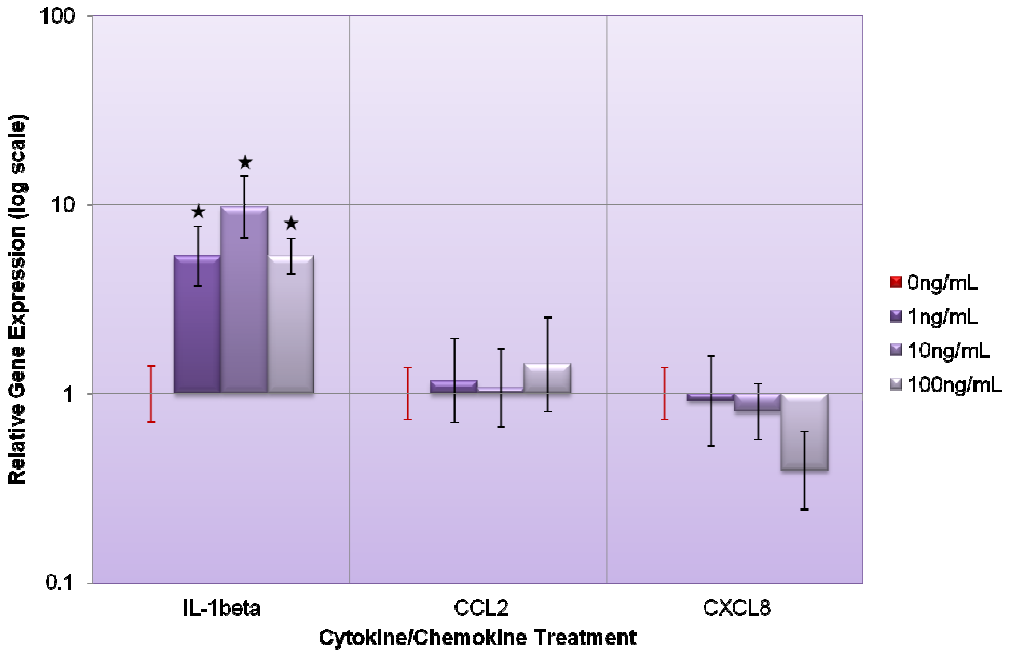


Figure 6-3 CCL2 mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each IL-1β treatment concentration was performed in triplicate on cultures derived from five different NP tissue samples (see **Table 2-8** for details of samples used). Each CCL2 and CXCL8 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). CCL2 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

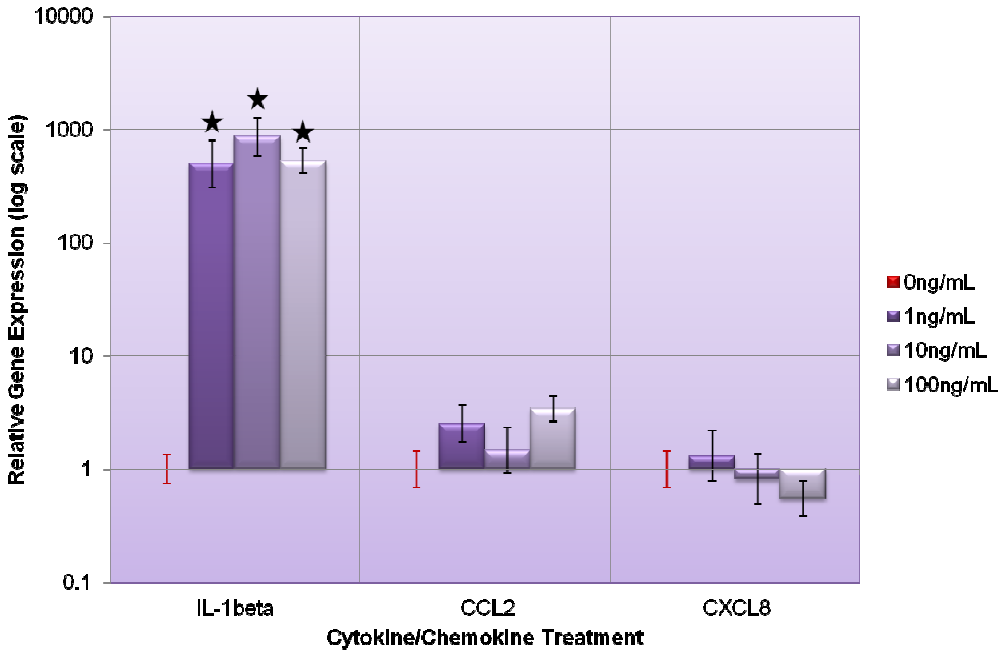


Figure 6-4 CXCL8 mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each IL-1β treatment concentration was performed in triplicate on cultures derived from five different NP tissue samples (see **Table 2-8** for details of samples used). Each CCL2 and CXCL8 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). CXCL8 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

shown). Treatment at 1ng/mL resulted in a 496 fold increase (*P*<0.0001), treatment at 10ng/mL resulted in an 862 fold increase (*P*<0.0001) and treatment at 100ng/mL resulted in a 527 fold increase (*P*<0.0001) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No alteration in CXCL8 mRNA expression pattern was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10 and 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-4).

6.2.2 Cytokine and Chemokine Gene Expression in Alginate Cultures

6.2.2.1 IL-1β mRNA Expression

Expression of IL-1β mRNA in alginate cultured NP cells under basal conditions was not observed in cells derived from the 5 IVDs investigated.

48 hour IL-1β treatment induced IL-1β expression and up-regulation in alginate cultured NP cells (Figure 6-5). Induction and up-regulation of IL-1β mRNA expression was observed separately in cells derived from the 3 IVDs subjected to treatment (data not shown). In all cases treatment at 1pg/mL was not sufficient to induce IL-1β mRNA expression. In cells derived from one IVD, treatment at 10pg/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 1.60; *P*>0.05). In all cases, treatment at 100pg/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 736.70; *P*<0.0001), treatment at 1ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 844.97; *P*<0.0001), treatment at 10ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 978.77; *P*<0.0001) and treatment at 100ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 1621.41; *P*<0.0001). Differences in mRNA expression levels between treatment groups were not significant.

Treatment with IL-16, CCL3 or CCL7 at 1, 10 or 100ng/mL was not seen to induce IL-1β mRNA expression in NP cells derived from the 3 IVD investigated (Figure 6-5).

6.2.2.2 IL-16 mRNA Expression

Expression of IL-16 mRNA in alginate cultured NP cells was observed under basal conditions in cells derived from 6 IVDs out of a total of 9 that were investigated.

In NP cells derived from 5 IVDs where basal IL-16 mRNA expression was observed, 48 hour IL-1β treatment resulted in down-regulation of IL-16 mRNA expression. Down-regulation of IL-16 mRNA expression was observed separately in cells derived from the 5 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 5.17 fold decrease (*P*>0.05), treatment at 10pg/mL resulted in a 5.14 fold decrease (*P*>0.05), treatment at 100pg/mL resulted in a 6.49 fold

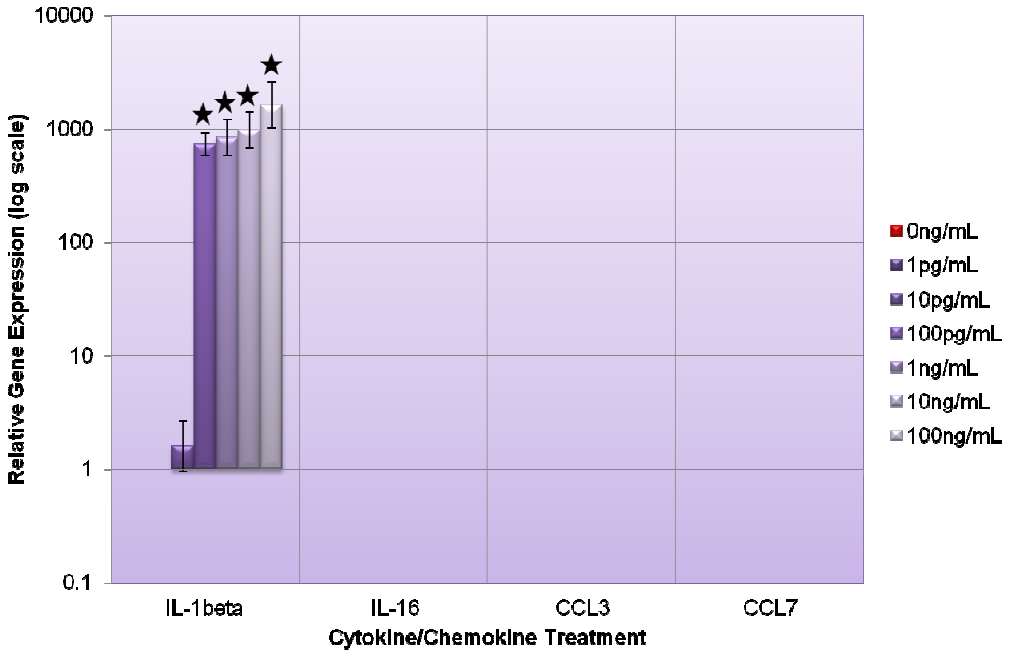


Figure 6-5 IL-1β mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see **Table 2-8** for details of samples used). IL-1β mRNA was not detected under basal conditions in any culture investigated although expression was induced following IL-1β treatment. Graphical data represents mean fold change in mRNA expression under treatment conditions normalised to internal reference gene expression (± SEM representation). ★ indicates $P<0.05$.

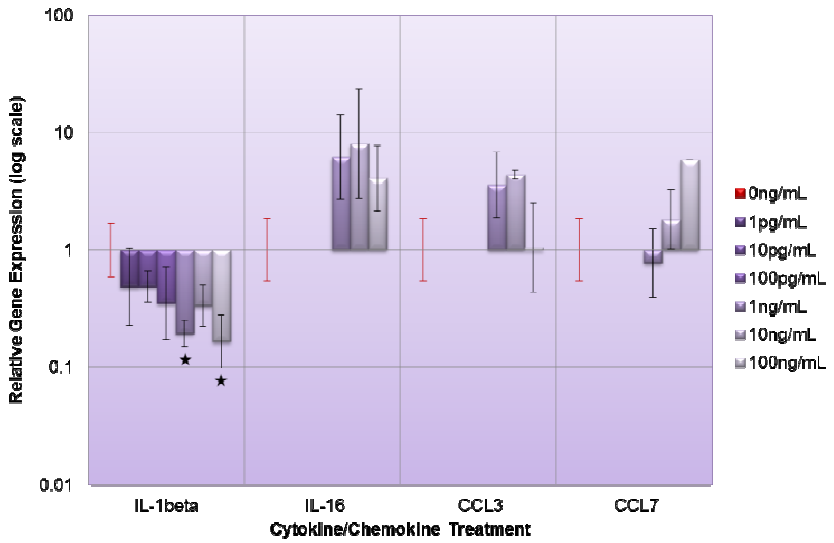


Figure 6-6 IL-16 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each IL-1β treatment concentration was performed in triplicate on cultures derived from seven different NP tissue samples (see Table 2-8 for details of samples used). Each IL-16, CCL3 and CCL7 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). IL-16 mRNA was detected under basal conditions in cultures derived from 5 of the 7 tissue samples subjected to IL-1β treatment, and in cultures derived from 1 of the 3 tissue samples subjected to IL-16, CCL3 and CCL7 treatment. Where IL-16 mRNA was detected under basal conditions data is shown and represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

decrease (*P*>0.05), treatment at 1ng/mL resulted in a 8.07 fold decrease (*P*=0.007), treatment at 10ng/mL resulted in a 6.68 fold decrease (*P*>0.05) and treatment at 100ng/mL resulted in a 8.35 fold decrease (*P*=0.0242) in mRNA expression compared to untreated controls. Differences in mRNA expression levels between treatment groups were not significant with the exception of 1ng/mL treatment that resulted in significantly decreased expression compared to 1pg/mL treatment (*P*=0.0174). In NP cells derived from 2 IVDs where basal IL-16 mRNA expression was not observed, 48 hour IL-1β treatment did not induce expression.

In NP cells derived from 3 IVDs where basal IL-16 mRNA expression was observed, 48 hour IL-16 treatment resulted in up-regulation of IL-16 mRNA expression although this was not significant (Figure 6-6). Up-regulation was observed separately in NP cells derived from the 3 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in a 6.16 fold increase (*P*>0.05), treatment at 10ng/mL resulted in an 8.00 fold increase (*P*>0.05) and treatment at 100ng/mL resulted in a 4.07 fold increase (*P*>0.05) in mRNA expression compared to untreated controls. In NP cells derived from 2 IVDs where basal expression was not observed, 48 hour IL-16 treatment did not induce IL-16 mRNA expression.

In NP cells derived from 1 IVD where basal IL-16 mRNA expression was observed, 48 hour CCL3 or CCL7 treatment had no significant effect on IL-16 mRNA expression (Figure 6-6). In NP cells derived from 2 IVDs where basal IL-16 mRNA expression was not observed, CCL3 or CCL7 treatment did not induce IL-16 mRNA expression.

6.2.2.3 CCL2 mRNA Expression

Constitutive CCL2 mRNA expression was observed under basal conditions in alginate cultured NP cells derived from the 5 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating CCL2 mRNA expression (Figure 6-7). Up-regulation of CCL2 mRNA expression was observed separately in cells derived from the 3 IVDs subjected to treatment (data not shown). Treatment at 1pg/mL resulted in a 3.9 fold increase (*P*=0.0099), treatment at 10pg/mL resulted in a 2.84 fold increase (*P*=0.0252), treatment at 100pg/mL resulted in a 6.76 fold increase (*P*=0.003), treatment at 1ng/mL resulted in a 6.03 fold increase (*P*=0.0018), treatment at 10ng/mL resulted in a 5.94 fold increase (*P*=0.0017) and treatment at 100ng/mL resulted in a 5.90 fold increase (*P*=0.0006) in CCL2 mRNA expression compared to untreated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in CCL2 mRNA expression pattern was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 or 100ng/mL in alginate cultured NP cells derived from the 3 IVDs investigated (Figure 6-7).

6.2.2.4 CCL3 mRNA Expression

Expression of CCL3 mRNA in alginate cultured NP cells under basal conditions was not observed in cells derived from the 5 IVDs investigated.

48 hour IL-1β treatment induced CCL3 mRNA expression and up-regulation in alginate cultured NP cells (Figure 6-8). Induction and up-regulation of CCL3 mRNA expression was observed separately in cells derived from the 3 IVDs subjected to treatment (data not shown). In all cases treatment at 1pg/mL was not sufficient to induce CCL3 mRNA expression. In cells derived from 2 IVDs, treatment at 10pg/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 2.51; *P*>0.05). In all cases, treatment at 100pg/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 61.63; *P*<0.0001), treatment at 1ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 75.92; *P*<0.0001), treatment at 10ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 78.44; *P*<0.0001) and treatment at 100ng/mL induced mRNA expression ($2^{-\Delta CT}$ x10^6 = 115.66; *P*<0.0001). A dose-dependent response to IL-1β treatment was observed with mRNA expression following 100pg/mL and 1, 10 and 100 ng/mL treatment significantly increased compared to 10pg/mL treatment (*P*=0.0006, *P*=0.0011, *P*=0.0016 and *P*=0.0079 respectively).

Treatment with IL-16, CCL3 or CCL7 at 1, 10 or 100ng/mL was not seen to induce CCL3 mRNA expression in NP cells derived from the 3 IVDs investigated (Figure 6-8).

6.2.2.5 CCL7 mRNA Expression

Constitutive CCL7 mRNA expression was observed under basal conditions in alginate cultured NP cells derived from the 5 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating CCL7 mRNA expression (Figure 6-9). Up-regulation of CCL7 mRNA was observed separately in cells derived from the 4 IVDs subjected to treatment (data not shown). Treatment at 1pg/mL resulted in a 1.64 fold increase (*P>0.05*), treatment at 10pg/mL resulted in a 2.50 fold increase (*P>0.05*), treatment at 100pg/mL resulted in a 3.52 fold increase (*P*=0.017), treatment at 1ng/mL resulted in a 2.99 fold increase (*P*=0.0327), treatment at 10ng/mL resulted in a 3.56 fold increase (*P*=0.0213) and treatment at 100ng/mL resulted in a 6.35 fold increase (*P*=0.0027) in CCL7 mRNA expression compared to untreated controls. Differences in expression level between treatment groups were not significant with

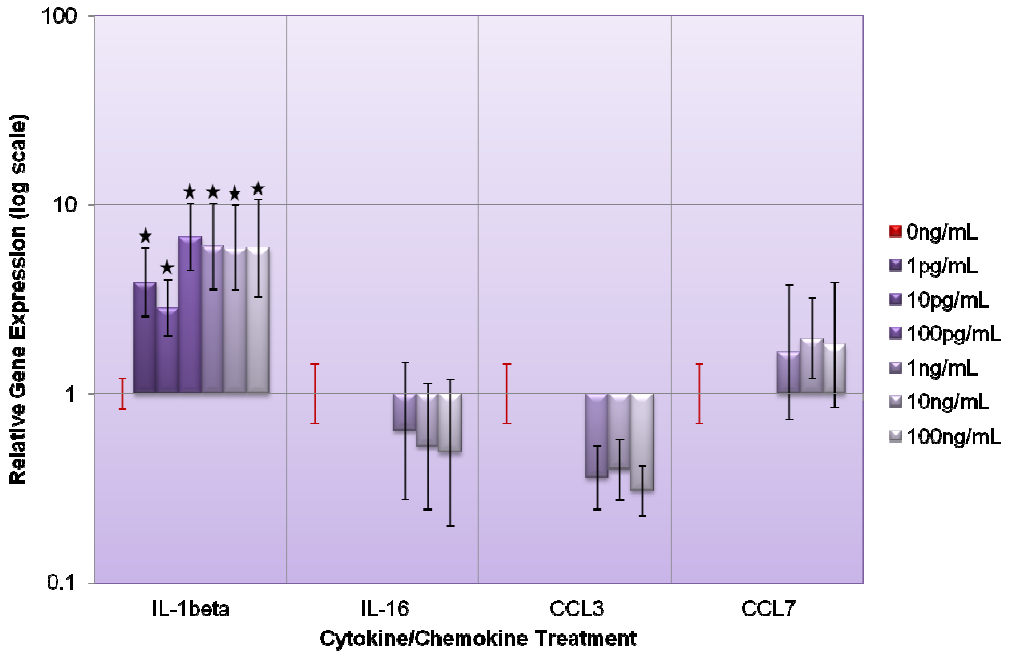


Figure 6-7 CCL2 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see **Table 2-8** for details of samples used). CCL2 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

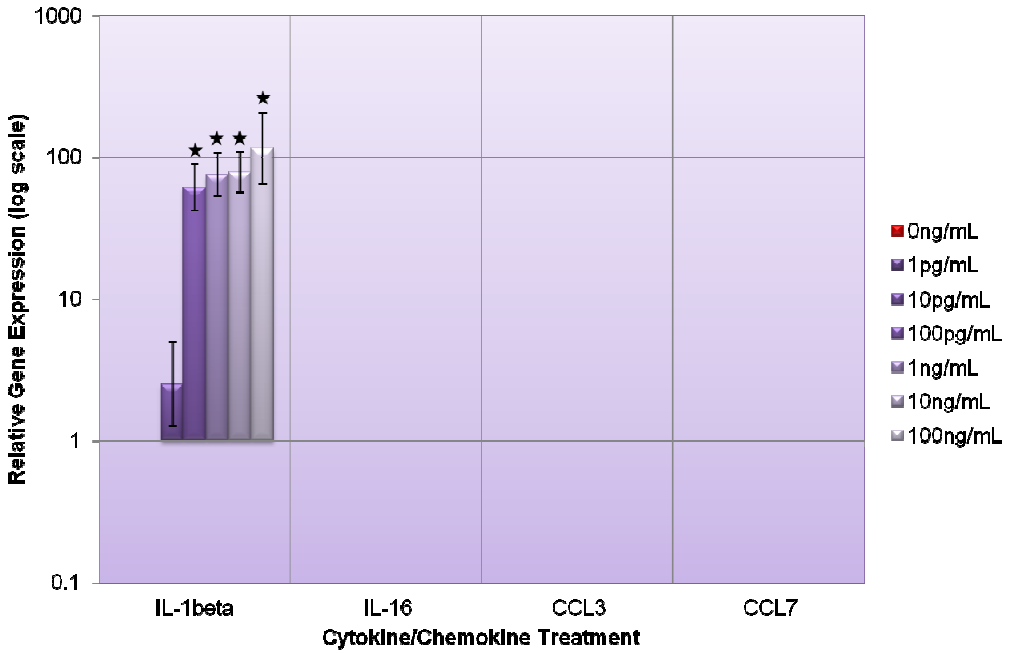


Figure 6-8 CCL3 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see **Table 2-8** for details of samples used). CCL3 mRNA was not detected under basal conditions in any culture investigated although expression was induced following IL-1β treatment. Graphical data represents mean fold change in mRNA expression under treatment conditions normalised to internal reference gene expression (± SEM representation). ★ indicates $P<0.05$.

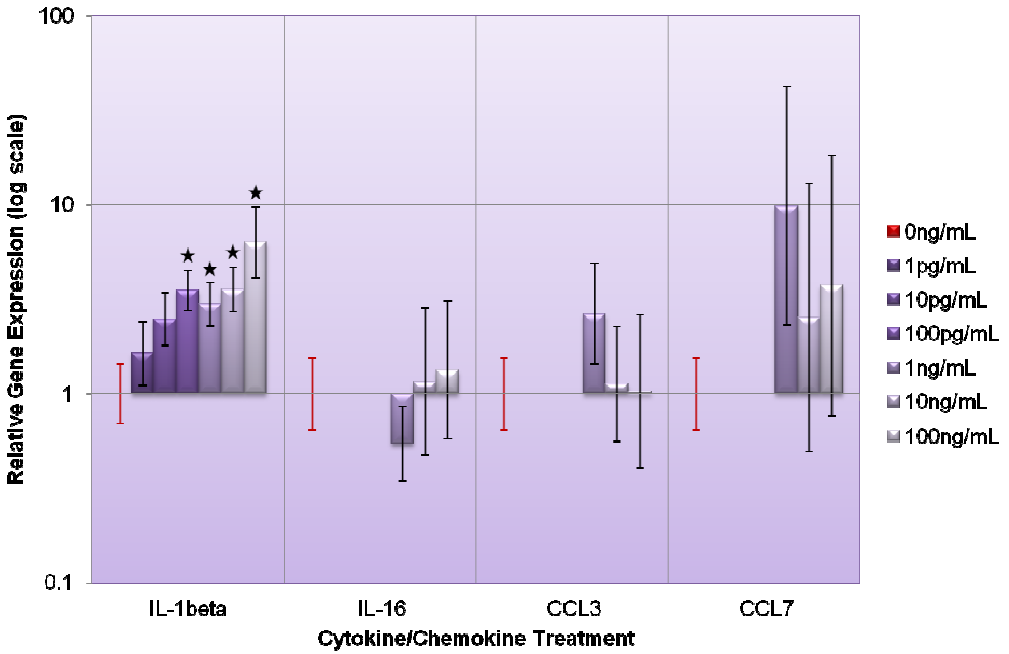


Figure 6-9 CCL7 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each IL-1β treatment concentration was performed in triplicate on cultures derived from four different NP tissue samples (see Table 2-8 for details of samples used). Each IL-16, CCL3 and CCL7 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see **Table 2-8** for details of samples used). CCL7 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

the exception of 100ng/mL treatment which was increased compared to 1pg/mL treatment (P=0.0388).

No significant alteration in CCL7 mRNA expression pattern was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 or 100ng/mL in alginate cultured NP cells derived from the 3 IVDs investigated (Figure 6-9).

6.2.2.6 CXCL8 mRNA Expression

Constitutive CXCL8 mRNA expression was observed under basal conditions in alginate cultured NP cells derived from the 5 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating CXCL8 mRNA expression (Figure 6-10). Up-regulation of CXCL8 mRNA was observed separately in cells derived from the 3 IVDs subjected to treatment (data not shown). Treatment at 1pg/mL resulted in a 12.65 fold increase (*P*=0.0503), treatment at 10pg/mL resulted in a 82.72 fold increase (*P*<0.0001), treatment at 100pg/mL resulted in a 834 fold increase (*P*<0.0001), treatment at 1ng/mL resulted in a 827 fold increase (*P*<0.0001), treatment at 10ng/mL resulted in a 844 fold increase (*P*<0.0001) and treatment at 100ng/mL resulted in a 909 fold increase (*P*<0.0001) in CXCL8 mRNA expression compared to untreated controls. A dose-dependent response was observed at treatment concentrations of up to 100pg/mL, with 10pg/mL treatment resulting in a significant up-regulation of mRNA expression compared to 1pg/mL (*P*=0.0151) and 100pg/mL treatment resulting in up-regulation compared to both 1pg/mL (*P*<0.0001) and 10pg/mL (*P*=0.0005). No significant differences were observed between treatment groups at concentrations 100pg/mL and above.

No significant alteration in CXCL8 mRNA expression was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 or 100ng/mL in alginate cultured NP cells derived from the 3 IVDs investigated (Figure 6-10).

6.2.3 Extracellular Matrix Gene Expression in Monolayer Cultures

6.2.3.1 Aggrecan mRNA Expression

Constitutive mRNA expression of aggrecan was observed in monolayer cultured NP cells derived from the 3 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by down-regulating aggrecan mRNA expression (Figure 6-11). Down-regulation of aggrecan mRNA expression was observed separately in cells derived from all 3 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in an 8.59 fold decrease (*P*=0.014), treatment at 10ng/mL resulted in a 7.97 fold decrease (*P*=0.0165) and treatment at 100ng/mL resulted in a 8.54 fold decrease (*P*=0.008)

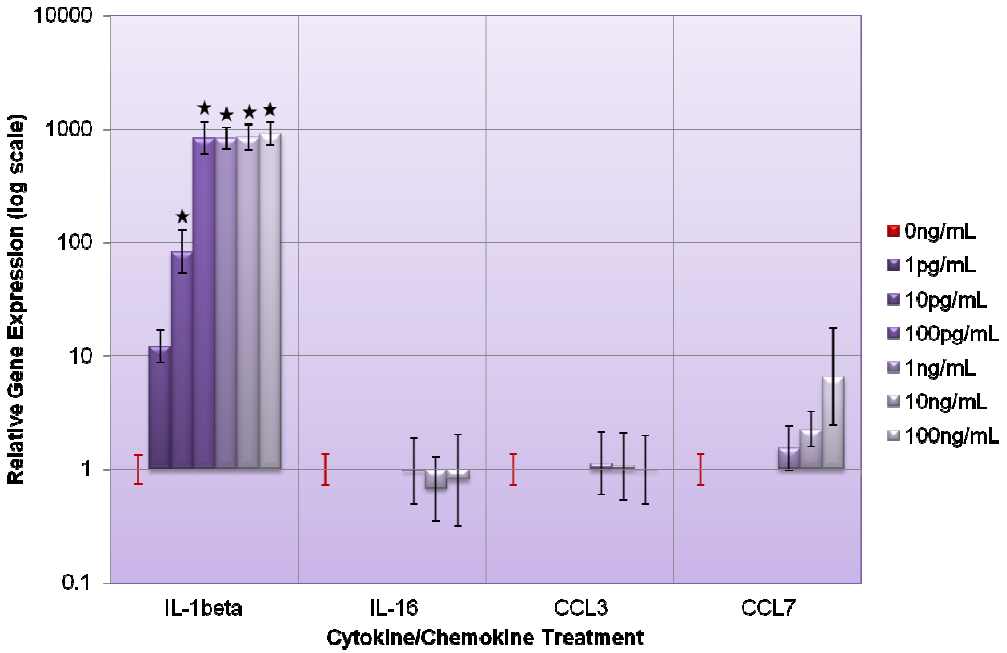


Figure 6-10 CXCL8 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see **Table 2-8** for details of samples used). CXCL8 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

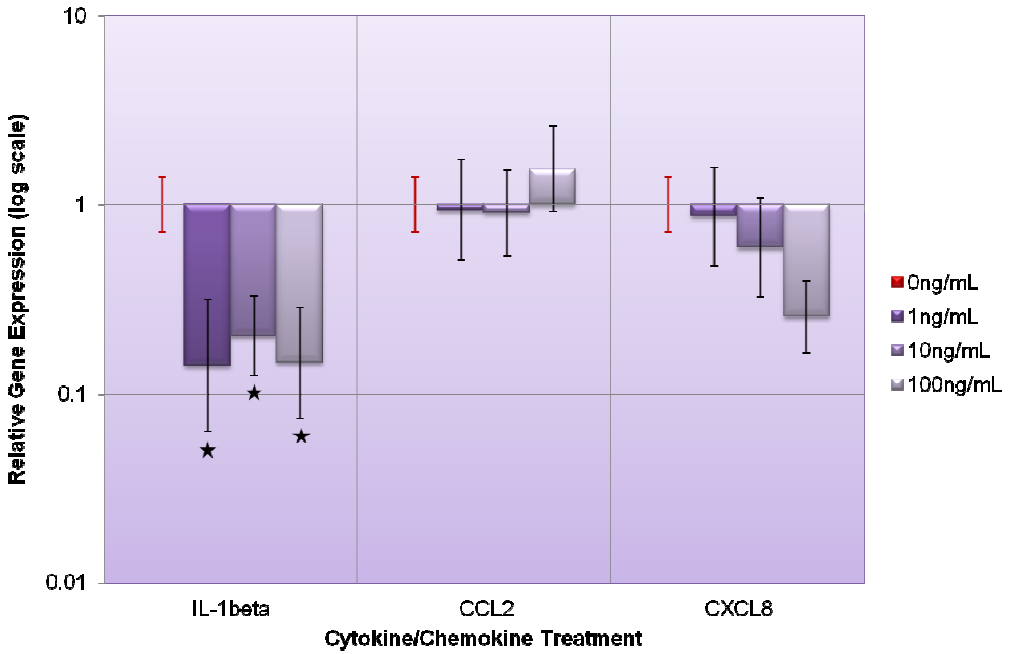


Figure 6-11 Aggrecan mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). Aggrecan mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in aggrecan mRNA expression was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10, or 100ng/mL in monolayer cultured NP cells derived from the 3 IVDs investigated (Figure 6-11). Although a 7.44 fold decrease in aggrecan mRNA expression was seen following 100ng/mL CXCL8 treatment however, this was not significant (P=0.0593).

6.2.3.2 MMP-3 mRNA Expression

Constitutive mRNA expression of MMP-3 was observed in monolayer cultured NP cells derived from the 3 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by up-regulating MMP-3 mRNA expression (Figure 6-12). Up-regulation of MMP-3 mRNA expression was observed separately in cells derived from all 3 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in a 22.27 fold increase (P<0.0001), treatment at 10ng/mL resulted in a 36.14 fold increase (P<0.0001) and treatment at 100ng/mL resulted in a 32.18 fold increase (P<0.0001) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in MMP-3 mRNA expression was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10 or 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-12).

6.2.3.3 MMP-13 mRNA Expression

Constitutive mRNA expression of MMP-13 was observed in monolayer cultured NP cells derived from the 3 IVDs investigated.

Monolayer cultured NP cells responded to 48 hour IL-1β treatment by up-regulating MMP-13 mRNA expression (Figure 6-13). Up-regulation of MMP-13 mRNA expression was observed separately in cells derived from all 3 IVDs investigated (data not shown). Treatment at 1ng/mL resulted in a 2.89 fold increase (P>0.05), treatment at 10ng/mL resulted in a 4.65 fold increase (P=0.0469) and treatment at 100ng/mL resulted in a 3.53 fold increase (P>0.05) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in MMP-13 mRNA expression was observed following 48 hour CCL2 or CXCL8 treatment at 1, 10 or 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-13).

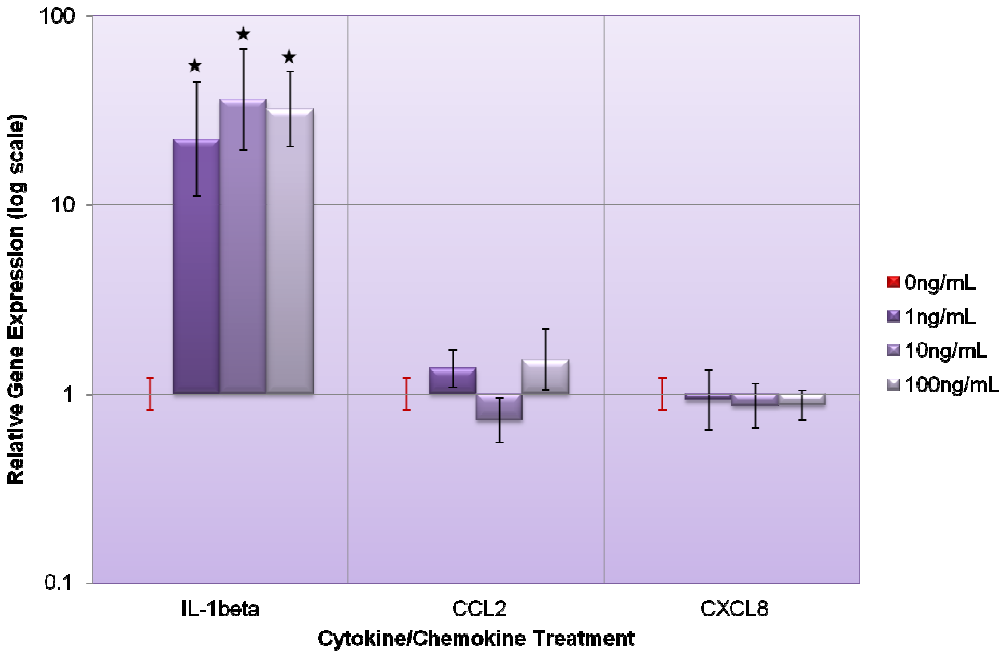


Figure 6-12 MMP-3 mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). MMP-3 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

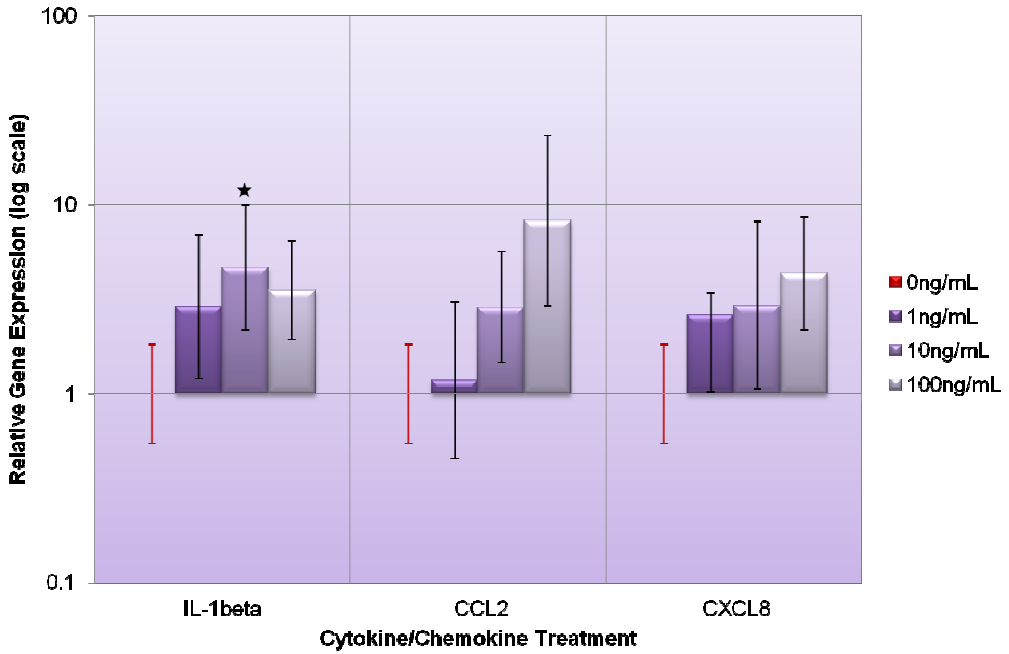


Figure 6-13 MMP-13 mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). MMP-13 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

6.2.3.4 ADAMTS-4 mRNA Expression

Constitutive mRNA expression of ADAMTS-4 was observed in monolayer cultured NP cells derived from the 3 IVDs investigated.

No significant alteration in ADAMTS-4 mRNA expression was observed following 48 hour IL-1β, CCL2 or CXCL8 treatment at 1, 10 or 100ng/mL in monolayer cultured cells derived from the 3 IVDs investigated (Figure 6-14).

6.2.4 Extracellular Matrix Gene Expression in Alginate Cultures

6.2.4.1 Aggrecan mRNA Expression

Constitutive mRNA expression of aggrecan was observed in alginate cultured NP cells derived from the 7 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment with a biphasic aggrecan mRNA expression pattern (Figure 6-15). Up-regulation of aggrecan mRNA expression following low-dose treatment and down-regulation of aggrecan mRNA expression following high-dose treatment was observed separately in cells derived from all 7 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 2.61 fold increase (P>0.05), treatment at 10pg/mL resulted in a 3.82 fold increase (P=0.063), treatment at 100pg/mL resulted in a 4.39 fold increase (P=0.0179) in mRNA expression compared to untreated controls. Following treatment at 1ng/mL, aggrecan mRNA expression had returned to basal expression levels, treatment at 10ng/mL resulted in a 5.24 fold decrease (P>0.05) and treatment at 100ng/mL resulted in an 8.87 fold decrease (P=0.0245) in mRNA expression compared to un-treated controls. Differences between treatment groups were observed with expression levels following 1pg/mL treatment significantly altered compared to treatment at 10ng/mL (P=0.0242) and 100ng/mL (P=0.0011). Expression following treatment at 10pg/mL was altered compared to treatment at 10ng/mL (P=0.0035) and 100ng/mL (P=<0.0001). Expression following treatment at 100pg/mL was altered compared to treatment at 1ng/mL (P=0.0416), 10ng/mL (P=0.0007) and 100ng/mL (P<0.0001). Expression following 1ng/mL treatment was altered compared to treatment at 100ng/mL (P=0.0093).

No significant alteration in aggrecan mRNA expression was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 and 100ng/mL in alginate cultured cells derived from the 3 IVDs investigated (Figure 6-15).

6.2.4.2 Type II Collagen mRNA Expression

Expression of type II collagen mRNA in alginate cultured NP cells was observed under basal conditions in cells derived from 3 IVDs, a total of 4 IVDs were investigated.

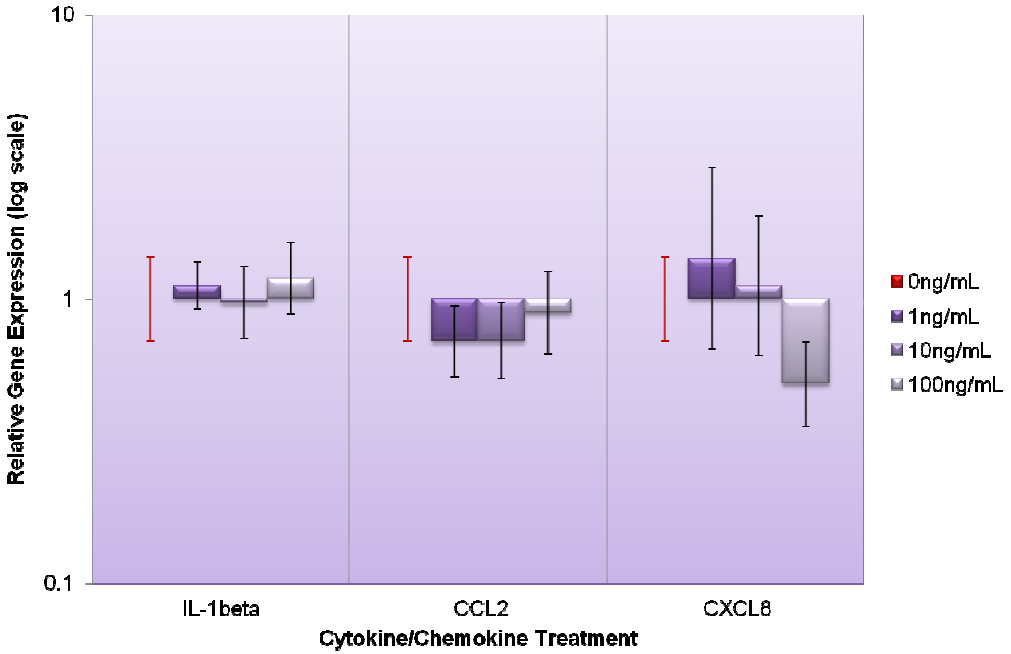


Figure 6-14 ADAMTS-4 mRNA Expression in Monolayer Cultured Nucleus Pulposus Cells Following IL-1β, CCL2 or CXCL8 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1, 10 or 100ng/mL IL-1β, CCL2 or CXCL8. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). ADAMTS-4 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

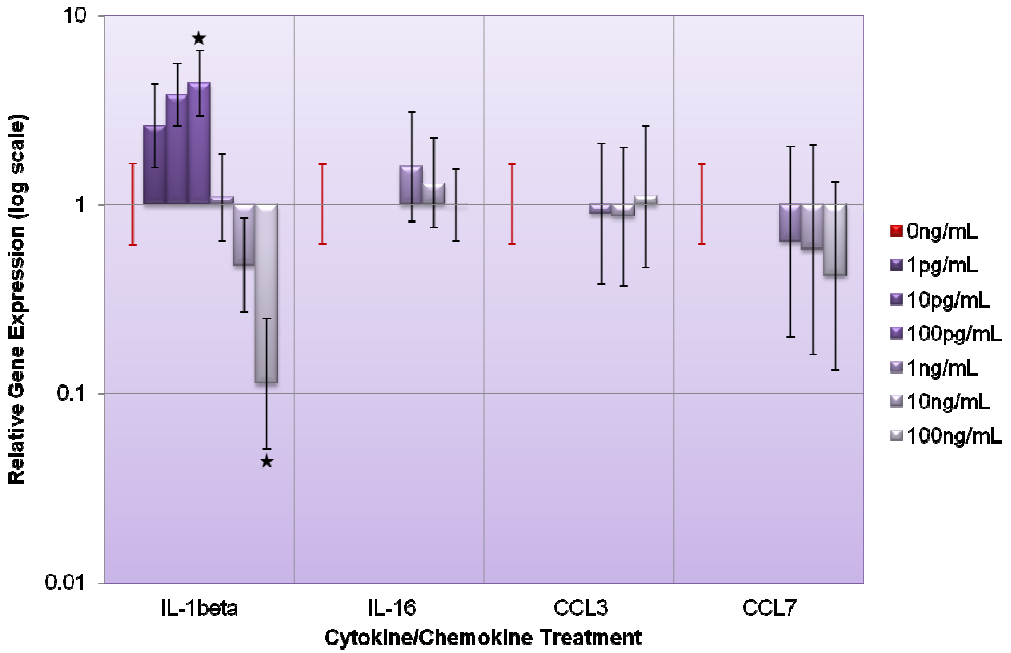


Figure 6-15 Aggrecan mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each IL-1β treatment concentration was performed in triplicate on cultures derived from seven different NP tissue samples (see Table 2-8 for details of samples used). Each IL-16, CCL3 and CCL7 treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). Aggrecan mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

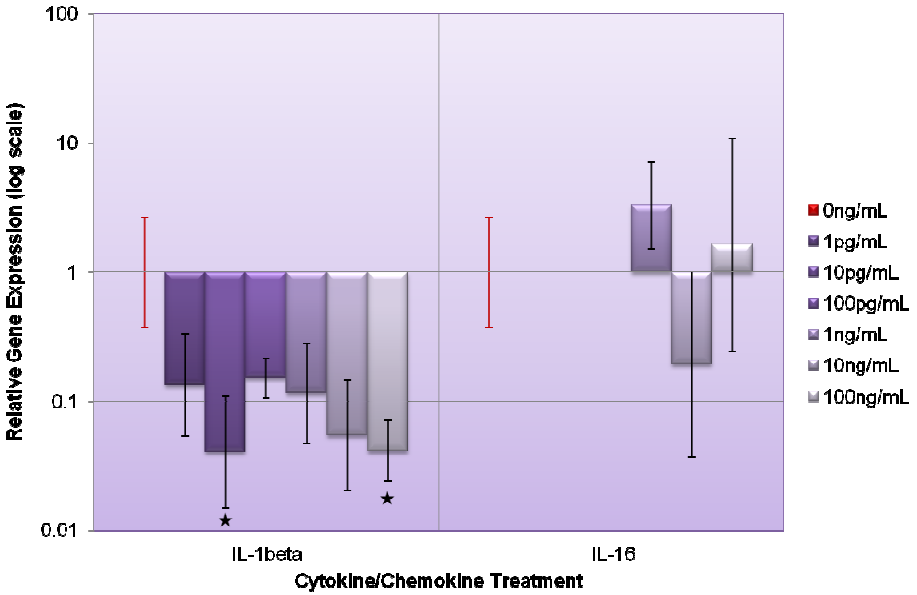


Figure 6-16 Type II Collagen mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β or IL-16 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16. Each IL-1β treatment concentration was performed in triplicate on cultures derived from seven different NP tissue samples (see Table 2-8 for details of samples used). Each IL-16 treatment concentration was performed in triplicate on cultures derived from six different NP tissue samples (see Table 2-8 for details of samples used). Type II collagen mRNA was detected under basal conditions in cultures derived from three of the seven tissue samples subjected to IL-1β treatment and two of the six samples subjected to IL-16 treatment. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

In NP cells derived from the 3 IVDs where basal expression was observed, 48 hour IL-1β treatment resulted in down-regulation of type II collagen mRNA (Figure 6-16). Down-regulation of type II collagen mRNA was observed separately in NP cells derived from all 3 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 8.66 fold decrease (P>0.05), treatment at 10pg/mL resulted in a 9.60 fold decrease (P=0.0486), treatment at 100pg/mL resulted in a 8.48 fold decrease (P>0.05), treatment at 1ng/mL resulted in a 8.85 fold decrease (P>0.05), treatment at 10ng/mL resulted in a 9.46 fold decrease (P>0.05) and treatment at 100ng/mL resulted in a 9.59 fold decrease (P=0.0248) in mRNA expression compared to untreated controls. Differences in mRNA expression levels between treatment groups were not significant. In NP cells derived from 1 IVD where basal expression was not observed, 48 hour IL-1β treatment did not induce type II collagen mRNA expression.

In NP cells derived from 2 IVDs where basal type II collagen mRNA expression was observed, 48 hour IL-16 treatment at 1, 10 or 100ng/mL had no significant effect on mRNA expression. In NP cells derived from 1 IVD where basal expression was not observed, 48 hour IL-16 treatment did not induce type II collagen mRNA expression (Figure 6-16).

6.2.4.3 MMP-3 mRNA Expression

Constitutive mRNA expression of MMP-3 was observed in alginate cultured NP cells derived from the 3 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating MMP-3 mRNA expression (Figure 6-17). Up-regulation of MMP-3 mRNA expression was observed separately in cells derived from all 3 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 12.38 fold increase (P>0.05), treatment at 10pg/mL resulted in a 159 fold increase (P=0.0007), treatment at 100pg/mL resulted in a 1779 fold increase (P<0.0001), treatment at 1ng/mL resulted in a 1068 fold increase (P<0.0001), treatment at 10ng/mL resulted in a 1383 fold increase (P<0.0001) and treatment at 100ng/mL resulted in a 1931 fold increase (P<0.0001) in mRNA expression compared to un-treated controls. The up-regulatory response was seen to be dose dependent at treatment concentrations of 100pg/mL and below, with mRNA expression following 10pg/mL treatment increased compared to 1pg/mL treatment (P=0.0139), and 100pg/mL treatment increased compared to both 1 and 10pg/mL treatments (P<0.0001 and P<0.0001 respectively). Differences in mRNA expression levels between 100pg/mL, 1, 10 and 100ng/mL treatment groups were not significant.

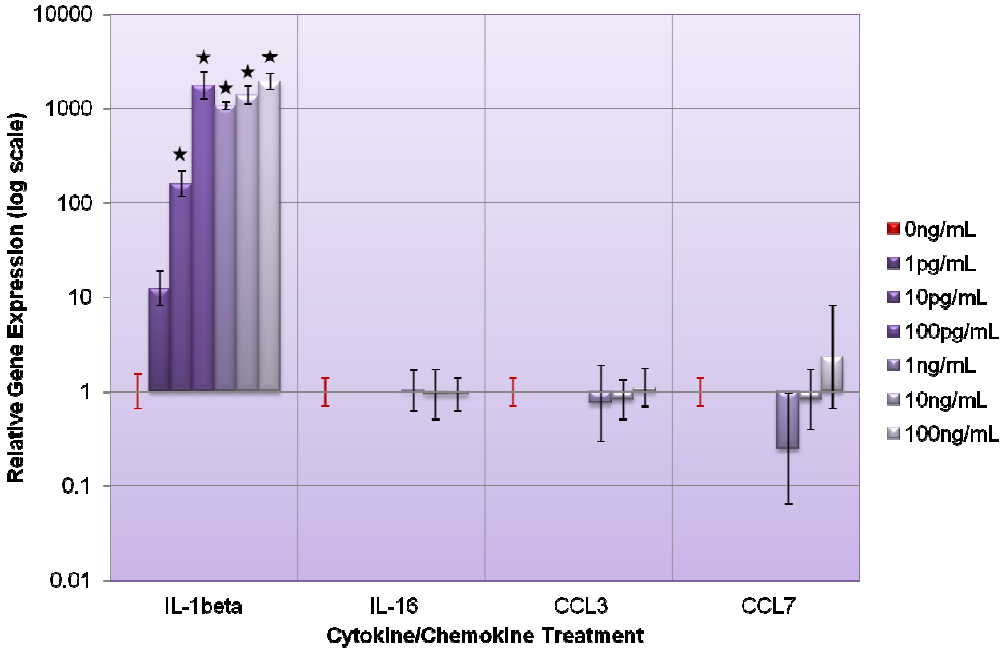


Figure 6-17 MMP-3 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). MMP-3 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

No significant alteration in MMP-3 mRNA expression was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 or 100ng/mL in alginate cultured cells derived from the 3 IVDs investigated (Figure 6-17).

6.2.4.4 MMP-13 mRNA Expression

Constitutive mRNA expression of MMP-13 was observed in alginate cultured NP cells derived from the 3 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating MMP-13 mRNA expression (Figure 6-18). Up-regulation of MMP-13 mRNA expression was observed separately in cells derived from all 3 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 42.18 fold increase (P=0.0003), treatment at 10pg/mL resulted in a 17.40 fold increase (P=0.0118) and treatment at 100pg/mL resulted in a 61.77 fold increase (P<0.0001), treatment at 1ng/mL resulted in a 71.57 fold increase (P<0.0001), treatment at 10ng/mL resulted in a 73.11 fold increase (P<0.0001) and treatment at 100ng/mL resulted in a 36.70 fold increase (P<0.0001) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in MMP-13 mRNA expression was observed following 48 hour IL-16, CCL3 or CCL7 treatment at 1, 10 or 100ng/mL in alginate cultured cells derived from the 3 IVDs investigated (Figure 6-18).

6.2.4.5 ADAMTS-4 mRNA Expression

Constitutive mRNA expression of ADAMTS-4 was observed in alginate cultured NP cells derived from the 4 IVDs investigated.

Alginate cultured NP cells responded to 48 hour IL-1β treatment by up-regulating ADAMTS-4 mRNA expression (Figure 6-19). Up-regulation of ADAMTS-4 mRNA was observed separately in cells derived from all 4 IVDs investigated (data not shown). Treatment at 1pg/mL resulted in a 2.78 fold increase (P>0.05), treatment at 10pg/mL resulted in a 5.11 fold increase (P>0.05), treatment at 100pg/mL resulted in a 8.19 fold increase (P=0.0663), treatment at 1ng/mL resulted in a 10.10 fold increase (P=0.024), treatment at 10ng/mL resulted in a 12.12 fold increase (P=0.0512) and treatment at 100ng/mL resulted in a 5.68 fold increase (P>0.05) in mRNA expression compared to un-treated controls. Differences in mRNA expression levels between treatment groups were not significant.

No significant alteration in ADAMTS-4 mRNA expression was observed following 48 hour IL-16 treatment at 1, 10 or 100ng/mL in alginate cultured cells derived from the 3 IVDs investigated (Figure 6-19).

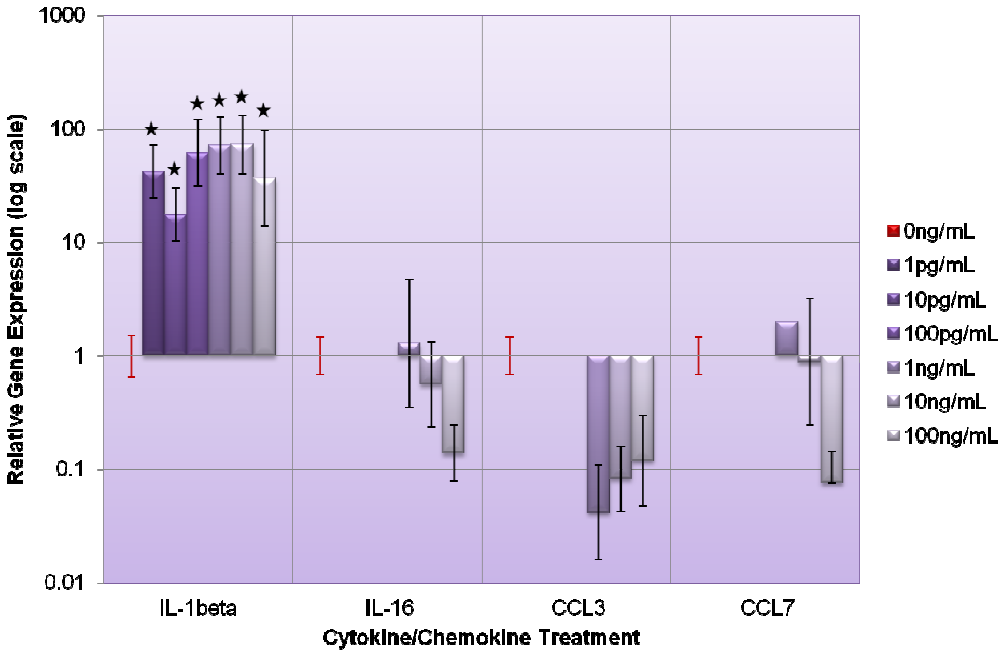


Figure 6-18 MMP-13 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β, IL-16, CCL3 or CCL7 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16, CCL3 or CCL7. Each treatment concentration was performed in triplicate on cultures derived from three different NP tissue samples (see Table 2-8 for details of samples used). MMP-13 mRNA was detected under basal conditions in all cultures investigated. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

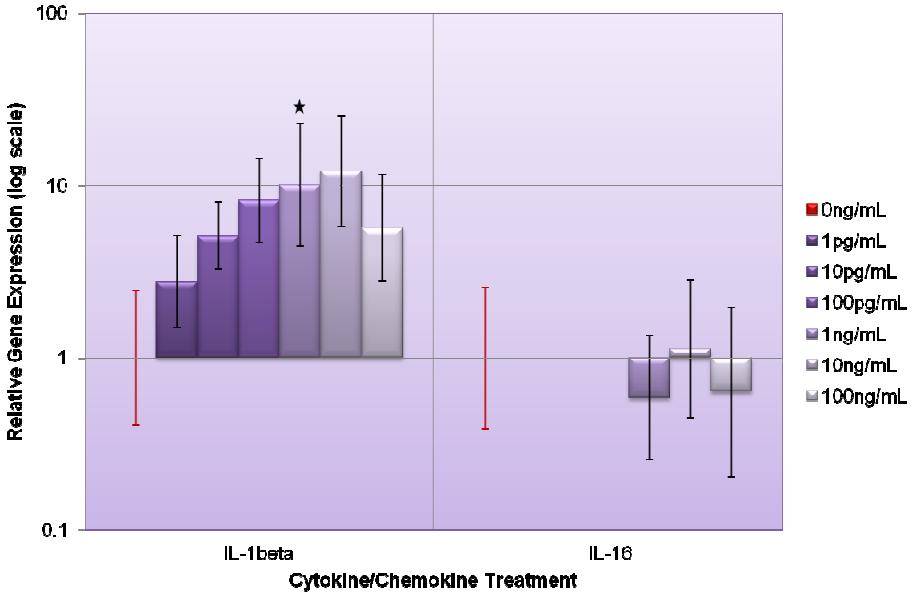


Figure 6-19 ADAMTS-4 mRNA Expression in Alginate Cultured Nucleus Pulposus Cells Following IL-1β or IL-16 Stimulation

Cultured NP cells were subjected to 48 hour treatment with 0, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β, or 0, 1, 10 or 100ng/mL IL-16. Each IL-1β treatment concentration was performed in triplicate on cultures derived from seven different NP tissue samples (see Table 2-8 for details of samples used). Each IL-16 treatment concentration was performed in triplicate on cultures derived from six different NP tissue samples (see Table 2-8 for details of samples used). ADAMTS-4 mRNA was detected under basal conditions in cultures derived from four of the seven tissue samples subjected to IL-1β treatment and three of the six samples subjected to IL-16 treatment. Graphical data represents mean fold change in mRNA expression under treatment conditions, normalised to both internal reference gene expression and target gene expression under basal conditions (± SEM representation). ★ indicates $P<0.05$.

6.2.5 Summary of Results

The results presented in this chapter are summarised in Table 6-1.

6.3 Discussion

The objective of this investigation was to determine the effects of cytokine and chemokine treatment on cytokine, chemokine, ECM component and ECM degrading enzyme gene expression. Data generated confirms the transcriptional modulatory potential of IL-1β on cytokine and chemokine gene expression, and on anabolic and catabolic gene expression. No significant transcriptional modulatory effects on gene expression were observed for IL-16, CCL2, CCL3, CCL7 or CXCL8 on NP cell biology for the parameters measured.

6.3.1 Modulation of Cytokine and Chemokine Gene Expression

In other arthropathies, master cytokines such as IL-1 and TNF modulate the expression of themselves and other factors, including cytokines and chemokines that are considered second step mediators of disease (Van den Berg & Miossec, 2004). Here, the modulatory potential of IL-1β on cytokine and chemokine gene expression by primary NP cells is demonstrated. IL-1β exerted modulatory potential over all cytokines and chemokines investigated, in both monolayer and alginate experimental systems.

IL-1β gene expression under basal conditions was detected in monolayer cultures and IL-1β treatment up-regulated this expression. Conversely, IL-1β gene expression was not detected in alginate cultures under basal conditions however, expression was induced by IL-1β treatment. Auto-induction and up-regulation of IL-1β gene expression has been described previously in primary NP cultures derived from degenerate IVDs (Le Maitre *et al.*, 2005). Further, it's reported that NP cells derived from non-degenerate IVDs respond to IL-1β treatment by down-regulating IL-1β gene expression (Le Maitre *et al.*, 2005). However, this modulatory effect was not observed here. NP cultures derived from prolapsed non-degenerate IVDs responded to IL-1β stimulation by up-regulating IL-1β gene expression similarly to NP cultures derived from prolapsed degenerate IVDs. This difference may be related to the origin of cells used and hence the pathology of prolapse, rather than degeneration, since previous investigations by Le Maitre *et al*. (2005) were performed on cultures derived from non-prolapsed non-degenerate IVDs.

Our findings in relation to the differential gene expression pattern of IL-1β under basal conditions in monolayer and alginate cultures suggest that phenotype, or differentiation state, exerts some influence over IL-1β gene expression by NP cells.

Table 6-1 Summary of IL-1β, IL-16, CCL2, CCL3, CCL7 and CXCL8 Treatment on Cytokine, Chemokine and Extracellular Matrix Component and Extracellular Matrix Degrading Enzyme Gene Expression

Target gene expression following 48 hour cytokine or chemokine treatment. ↑, increase; ↓ decrease; *, significant difference between basal expression levels and cytokine or chemokine stimulated expression levels (P<0.05)

Cytokine/Chemokine Target	Monolayer	Alginate	ECM Target	Monolayer	Alginate
IL-1β Treatment					
IL-1β	↑*	↑*	**Aggrecan**	↓*	↑*↓* (dose dependent)
IL-16	Not investigated	↓*	**Type II Collagen**	Not investigated	↓*
CCL2	↑*	↑*	**MMP-3**	↑*	↑*
CCL3	Not investigated	↑*	**MMP-13**	↑*	↑*
CCL7	Not investigated	↑*	**ADAMTS-4**	No change	↑*
CXCL8	↑*	↑*			
IL-16 Treatment					
IL-1β	Not investigated	Not detected	**Aggrecan**	Not investigated	No change
IL-16	Not investigated	↑	**Type II Collagen**	Not investigated	No change
CCL2	Not investigated	No change	**MMP-3**	Not investigated	No change
CCL3	Not investigated	Not detected	**MMP-13**	Not investigated	No change
CCL7	Not investigated	No change	**ADAMTS-4**	Not investigated	No change
CXCL8	Not investigated	No change			
CCL2 Treatment					
IL-1β	No change	Not investigated	**Aggrecan**	No change	Not investigated
IL-16	Not investigated	Not investigated	**Type II Collagen**	Not investigated	Not investigated

Cytokine/Chemokine Target	Monolayer	Alginate	ECM Target	Monolayer	Alginate
CCL2 Treatment					
CCL2	No change	Not investigated	**MMP-3**	No change	Not investigated
CCL3	Not investigated	Not investigated	**MMP-13**	↑	Not investigated
CCL7	Not investigated	Not investigated	**ADAMTS-4**	No change	Not investigated
CXCL8	No change	Not investigated			
CCL3 Treatment					
IL-1β	Not investigated	Not detected	**Aggrecan**	Not investigated	No change
IL-16	Not investigated	No change	**Type II Collagen**	Not investigated	Not detected
CCL2	Not investigated	↓	**MMP-3**	Not investigated	No change
CCL3	Not investigated	Not detected	**MMP-13**	Not investigated	↓
CCL7	Not investigated	No change	**ADAMTS-4**	Not investigated	Not detected
CXCL8	Not investigated	No change			
CCL7 Treatment					
IL-1β	Not investigated	Not detected	**Aggrecan**	Not investigated	No change
IL-16	Not investigated	No change	**Type II Collagen**	Not investigated	Not detected
CCL2	Not investigated	No change	**MMP-3**	Not investigated	No change
CCL3	Not investigated	Not detected	**MMP-13**	Not investigated	No change
CCL7	Not investigated	No change	**ADAMTS-4**	Not investigated	Not detected
CXCL8	Not investigated	↑			
CXCL8 Treatment					
IL-1β	No change	Not investigated	**Aggrecan**	↓	Not investigated
IL-16	Not investigated	Not investigated	**Type II Collagen**	Not investigated	Not investigated

Table 6-1 Continued from previous page.

Cytokine/Chemokine Target	Monolayer	Alginate	ECM Target	Monolayer	Alginate
CXCL8 Treatment					
CCL2	No change	Not investigated	**MMP-3**	No change	Not investigated
CCL3	Not investigated	Not investigated	**MMP-13**	↑	Not investigated
CCL7	Not investigated	Not investigated	**ADAMTS-4**	No change	Not investigated
CXCL8	No change	Not investigated			

Table 6-1 Continued from previous page.

This finding may also account for the difference between data presented here and Le Maitre *et al*.'s previous report (2005) of down-regulation of IL-1β transcription following IL-1β stimulation, in cultures derived from non-degenerate IVDs. Here, cultures expanded in monolayer were encapsulated in alginate and cultured for 14 days to allow re-establishment of native cytokine and chemokine gene expression profiles, in Le Maitre *et al*.'s previous investigation (2005) monolayer expanded cultures were encapsulated in alginate for 28 days prior to IL-1β stimulation. Hence, since differentiation state exerts some influence over IL-1β transcription these studies are not directly comparable, although both clearly demonstrate the up-regulatory effect of IL-1β stimulation on IL-1β transcription in cultures derived from degenerate IVDs.

The modulatory potential of IL-1β on IL-16 gene expression was investigated in alginate cultures. Interestingly, IL-16 expression was not detected in alginate cultures under basal conditions derived from all tissue samples investigated. Of the cultures investigated that were derived from 9 different tissue samples, only 6 displayed IL-16 mRNA expression under basal conditions. IL-16 expression in primary NP cultures may be related to tissue source and represent differences between individual tissue donors or previous modulatory events occurring *in vivo* prior to tissue harvest. Where IL-16 gene expression was observed, NP cultures responded to IL-1β stimulation by down-regulating IL-16 transcription.

This is the first report of IL-16 gene expression in NP cultures although gene expression has been shown previously in cultured primary human synovial fibroblasts derived from OA and RA tissue donors (Sciaky *et al.*, 2000). The effect of IL-1β stimulation on IL-16 transcript abundance in synovial fibroblasts was determined for treatment durations of up to 7 hours, although no alterations in transcript abundance were detected during this time frame (Sciaky *et al.*, 2000). However, IL-16 protein production was increased and the authors conclude that IL-1β stimulation induces translation of preformed IL-16 mRNA to increase expression of IL-16 protein (Sciaky *et al.*, 2000).

The modulation of CCL2 and CXCL8 gene expression by IL-1β was investigated in both monolayer and alginate culture systems. Similar expression patterns were observed between monolayer and alginate cultures and IL-1β stimulation up-regulated CCL2 and CXCL8 gene expression in both experimental systems. The up-regulatory effect of IL-1β on CCL2 and CXCL8 gene expression has been

described previously in primary NP cultures stimulated with 30ng/mL IL-1β (Huang *et al.*, 2008).

CCL3 and CCL7 gene expression was investigated in alginate cultures only. CCL3 gene expression was not detected under basal conditions although IL-1β treatment induced expression. CCL7 gene expression was detected under basal conditions and stimulation with IL-1β up-regulated this expression. Expression of CCL3 or CCL7 in primary NP cultures has not been investigated previously however, CCL3 expression has been investigated in cultured primary human articular chondrocytes (Yuan *et al.*, 2001; Sandell *et al.*, 2008). The effect of IL-1β stimulation on articular chondrocytes was induction of CCL3 gene expression at treatment concentrations of 100pg/mL and above (Sandell *et al.*, 2008), which is the same concentration required here to induce expression in primary NP cells.

The effects of IL-16 stimulation on cytokine and chemokine gene expression were investigated in alginate cultured NP cells. No significant modulatory effects were observed although a trend of auto up-regulation was observed in NP cultures that exhibited IL-16 gene expression under basal conditions. This may indicate an autocrine or paracrine signalling circuit for this ligand in primary NP cells. The effect of IL-16 stimulation on IL-1β transcription was not determined since alginate NP cultures did not exhibit IL-1β gene expression under basal conditions. However, IL-16 may have modulatory potential over IL-1β since it is demonstrated in animal models of RA that intra-articular injection of recombinant IL-16 reduces IL-1β transcription in associated pathological tissues (Klimiuk *et al.*, 1999).

The effects of CCL2 and CXCL8 stimulation on cytokine and chemokine gene expression were investigated in monolayer NP cultures only. No modulatory effects were observed for these chemokines and there are no previous reports of transcriptional regulatory potential for these molecules in any cell type. The effects of CCL3 and CCL7 stimulation on cytokine and chemokine gene expression were investigated in alginate cultures only. No significant transcriptional modulatory effects were observed for these chemokines although a trend of CCL2 down-regulation following CCL3 treatment and a trend of CXCL8 up-regulation following high dose (100ng/mL) CCL7 treatment was observed.

6.3.2 Modulation of ECM Gene Expression

In chronic inflammation or disease, prolonged or dysregulated cytokine production can drive the equilibrium of tissue homeostasis towards catabolism, which is associated with tissue destruction and degeneration (Dinarello & Moldawer, 2000). Here, IL-1β exerted transcriptional modulatory potential over all anabolic and

catabolic ECM genes investigated in a manner that suggests the effects of high concentrations of IL-1β in the extracellular milieu of native NP cells would be detrimental to ECM integrity. The effects of IL-1β stimulation were investigated in both monolayer and alginate experimental systems.

The modulatory potential of IL-1β stimulation on aggrecan gene expression was investigated in monolayer and alginate experimental systems. Monolayer cultures responded to IL-1β stimulation by down-regulating aggrecan transcription. This effect was investigated in monolayer cultures with IL-1β treatment concentrations of 1, 10 and 100ng/mL. Under these conditions the down-regulatory effect of IL-1β was not dose-dependent and the effect of treatment appeared maximal at the lowest treatment concentration (1ng/mL). In subsequent alginate culture investigations lower concentrations of IL-1β treatment (1, 10 and 100pg/mL) were run alongside those previously investigated in monolayer. The results of low-dose IL-1β stimulation of alginate cultures were interesting, revealing not only a dose-dependent response but a differential expression pattern dependent on treatment concentration, whereby low-dose (≤100pg/mL) induced up-regulation of aggrecan transcription and high-dose (>1ng/mL) induced down-regulation.

The effect of IL-1β stimulation on aggrecan transcription has been investigated previously in primary human NP cultures (Le Maitre *et al.*, 2005; Studer *et al.*, 2011). These studies demonstrate that cultures derived from both non-degenerate and degenerate IVDs respond to IL-1β stimulation by down-regulating aggrecan transcription (Le Maitre *et al.*, 2005; Studer *et al.*, 2011), although neither investigation used treatment concentrations of <10ng/mL. Modulation of aggrecan transcription by IL-1β is also described in cultured articular chondrocytes, where treatment concentrations between 0.5 and 10ng/mL have been investigated (Richardson & Dodge, 2000). Although the lowest treatment concentrations used in this investigation did not elicit a significant alteration in aggrecan transcription as was seen for higher treatment concentrations that down-regulated relative mRNA expression levels (Richardson & Dodge, 2000).

Data generated in this investigation confirms these previous reports of down-regulation of aggrecan transcription by high dose (≥10ng/mL) IL-1β stimulation (Richardson & Dodge, 2000; Le Maitre *et al.*, 2005; Studer *et al.*, 2011) in NP cultures derived from both non-degenerate (Le Maitre *et al.*, 2005) and degenerate IVDs (Studer *et al.*, 2011). However, here we present a novel finding in that this is the first report of a differential expression pattern dependent on stimulation dose.

Indeed, this data suggests that low levels of IL-1β, as has been identified within NP tissue samples derived from normal tissue donors (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d) may be beneficial to ECM integrity, by up-regulating aggrecan transcription.

The modulatory potential of IL-1β on type II collagen transcription was investigated in alginate cultures only. The rationale for excluding type II collagen from monolayer culture investigations was that NP cell transcription of type II collagen is altered dependent on differentiation state notably, decreased or absent from monolayer cultures that adopt a fibroblastic phenotype (Baer *et al.*, 2001; Wang *et al.*, 2001; Le Maitre *et al.*, 2005). Hence, data generated in relation to type II collagen gene expression in monolayer cultures may not be physiologically relevant.

Alginate NP cultures responded to IL-1β stimulation by down-regulating type II collagen transcription, significant modulation was observed following stimulation with as little as 10pg/mL. The effect of IL-1β stimulation on type II collagen transcription has been investigated previously in primary NP cultures (Le Maitre *et al.*, 2005; Studer *et al.*, 2011). These studies demonstrate a differential response to IL-1β stimulation dependent on tissue pathology, with IL-1β stimulation of NP cultures derived from non-degenerate IVDs eliciting no significant effect (Le Maitre *et al.*, 2005), whilst stimulation of cultures derived from degenerate IVDs results in down-regulation (Studer *et al.*, 2011). Here, the down-regulatory effect of IL-1β stimulation on type II collagen transcription was observed in NP cultures derived from prolapsed non-degenerate IVDs, similarly to that observed in NP cultures derived from prolapsed degenerate IVDs. This difference may again be related to the origin of the cells since previous investigations by Le Maitre *et al*. (2005) were performed on NP cultures derived from non-prolapsed non-degenerate IVDs.

Data generated in this investigation confirms previous reports of down-regulation of type II collagen transcription by high dose (≥10ng/mL) IL-1β stimulation in NP cultures derived from degenerate IVDs (Studer *et al.*, 2011). Further, we report the modulatory potential of low dose IL-1β stimulation (≤100pg/mL) on type II collagen transcription in NP cultures derived from prolapsed non-degenerate and prolapsed degenerate IVDs.

The modulatory potential of IL-1β stimulation on MMP-3 and -13 transcription was investigated in monolayer and alginate experimental systems. Monolayer cultures responded to IL-1β stimulation by up-regulating MMP-3 and -13 transcription. This

effect was investigated in monolayer cultures following stimulation with 1, 10 and 100ng/mL IL-1β. Under these conditions a dose-dependent response was not observed for either target, and for MMP-3 transcription the effect of treatment appeared maximal at the lowest treatment concentration (1ng/mL).

In subsequent alginate culture investigations lower concentrations of IL-1β treatment (1, 10 and 100pg/mL) were run alongside those previously investigated in monolayer. The results of low-dose IL-1β stimulation of alginate cultures revealed that the up-regulation of MMP-3 following IL-1β stimulation is dose-dependent at low treatment concentrations (≤100pg/mL). MMP-13 transcription following IL-1β stimulation was also seen to follow the same up-regulatory trend observed in monolayer cultures, although dose dependency was not observed even following low dose (≤100pg/mL) stimulation and the effect of treatment appeared maximal at the lowest treatment concentration (1pg/mL). Comparative analysis of equivalent IL-1β dose stimulation (1, 10 and 100ng/mL) between monolayer and alginate experimental systems indicates that whilst the same trend of up-regulation is observed, the response to stimulation is greater in alginate cultures. In monolayer cultures, fold increases of <40 were observed, whereas in alginate cultures fold increases of >1000 in MMP-3 transcription were observed. For MMP-13 transcription, in monolayer cultures fold increases of <5 were observed, whereas in alginate cultures fold increases of >35 were observed.

The modulatory potential of IL-1β on MMP-3 and -13 transcription has been investigated previously in primary human NP cultures (Le Maitre *et al.*, 2005; Millward-Sadler *et al*., 2009). These studies demonstrate that cultures derived from both non-degenerate and degenerate IVDs respond to IL-1β stimulation by up-regulating MMP-3 and -13 transcription (Le Maitre *et al.*, 2005; Millward-Sadler *et al*., 2009), although neither investigation used treatment concentrations of <10ng/mL. Modulation of MMP-3 and -13 transcription by IL-1β is also described in cultured articular chondrocytes, where treatment concentrations between 0.5 and 10ng/mL have been investigated (Richardson & Dodge, 2000). This study reports significant modulation of MMP-3 transcription at treatment concentrations of ≥1ng/mL and significant modulation of MMP-13 transcription at 10ng/mL treatment concentration in chondrocyte suspension cultures (Richardson & Dodge, 2000).

Data generated in this investigation confirms these previous reports of up-regulation of MMP-3 and -13 transcription by high dose (≥1ng/mL) IL-1β stimulation (Richardson & Dodge, 2000; Le Maitre *et al.*, 2005; Millward-Sadler *et al.*, 2009) in

NP cultures derived from both non-degenerate (Le Maitre *et al.*, 2005; Millward-Sadler *et al.*, 2009) and degenerate IVDs (Millward-Sadler *et al.*, 2009). Further, we report the modulatory potential of low dose IL-1β stimulation (≤100pg/mL) on MMP-3 and -13 transcription in NP cultures derived from prolapsed non-degenerate and prolapsed degenerate IVDs.

The modulatory potential of IL-1β stimulation on ADAMTS-4 transcription was investigated in monolayer and alginate experimental systems. Whilst gene expression of ADAMTS-4 mRNA was detected in monolayer cultures under basal conditions, transcription was not affected by IL-1β stimulation. However, in subsequent alginate culture investigations IL-1β stimulation up-regulated ADAMTS-4 transcription. The effect of IL-1β stimulation on ADAMTS-4 transcription has been investigated previously in primary human NP cultures (Le Maitre *et al.*, 2005). This study demonstrated a differential response to IL-1β stimulation dependent on tissue pathology, with IL-1β stimulation of NP cultures derived from non-degenerate IVDs eliciting no significant effect (Le Maitre *et al.*, 2005), whilst stimulation of cultures derived from degenerate IVDs resulted in up-regulation (Le Maitre *et al.*, 2005) of ADAMTS-4 transcription. Here, the up-regulatory effect of IL-1β stimulation in alginate cultures on ADAMTS-4 transcription was observed in NP cultures derived from prolapsed non-degenerate IVDs, similarly to that observed in NP cultures derived from prolapsed degenerate IVDs. This difference may again be related to the origin of the cells since previous investigations by Le Maitre *et al*. (2005) were performed on NP cultures derived from non-prolapsed non-degenerate IVDs.

Data generated in this investigation confirms this previous report of up-regulation of ADAMTS-4 transcription by IL-1β stimulation in NP cultures derived from degenerate IVDs (Le Maitre *et al.*, 2005). Further, we report the modulatory potential of IL-1β stimulation on ADAMTS-4 transcription in alginate NP cultures derived from prolapsed non-degenerate and prolapsed degenerate IVDs.

In relation to the transcriptional modulatory effects of IL-1β on ECM degrading enzymes, cell phenotype, or differentiation state appears to have some influence over the response elicited. For MMP-3 and -13, modulatory effects were greater in alginate cultures that are thought to more closely resemble the *in vivo* phenotype than in de-differentiated monolayer cultures. For ADAMTS-4, IL-1β had no modulatory potential over monolayer cultures but elicited an up-regulatory response in alginate cultures indicating that this response is phenotype dependent.

No significant modulatory effect on ECM gene expression was observed following 48 hour stimulation of primary NP cultures with IL-16, CCL2, CCL3, CCL7 or CXCL8. The effect of IL-16 stimulation on ECM gene expression has not been investigated previously in any cell type. However, for the chemokines investigated here, several reports of modulation have been made in articular chondrocytes.

The modulatory potential of CCL2 and CCL3 stimulation on proteoglycan synthesis has been investigated previously in primary human articular chondrocytes (Yuan *et al.*, 2001). This study demonstrated down-regulation of proteoglycan synthesis measured by incorporation of ^{35}S into newly synthesised proteoglycan molecules (Yuan *et al.*, 2001). Here, the effects of CCL2 and CCL3 stimulation on transcription of the IVD proteoglycan, aggrecan, was investigated in either monolayer or alginate experimental systems. Data generated indicates that CCL2 and CCL3 do not elicit modulatory effects on aggrecan transcription in primary NP cultures. However, since the investigations by Yuan *et al*. (2001) did not investigate modulatory effects of CCL2 and CCL3 stimulation on proteoglycan transcription, it is unknown whether the down-regulation of proteoglycan synthesis in articular chondrocytes is a transcriptional modulation or a translational modulation. Hence, potentially CCL2 and CCL3 may modulate proteoglycan synthesis by NP cells if their modulatory effect is translational.

The modulatory potential of CCL2 and CXCL8 stimulation on ECM degrading enzyme production has been investigated previously in primary articular chondrocytes (Borzi *et al.*, 2000; Yuan *et al.*, 2001). These studies demonstrate that CCL2 stimulation up-regulates MMP-3 transcription (Yuan *et al.*, 2001) and that CCL2 and CXCL8 stimulation up-regulate the release of MMP-3 from chondrocyte cultures (Borzi *et al.*, 2000; Yuan *et al.*, 2001). Here, the effects of CCL2 and CXCL8 stimulation were investigated in monolayer cultures. Data generated indicates that CCL2 and CXCL8 do not elicit modulatory effects on MMP-3 transcription in primary NP cultures. However, since protein level investigations were not performed here these chemokines may modulate release of ECM degrading enzymes.

The data presented here indicates clearly that IL-1β exerts significant transcriptional modulatory potential over cytokines, chemokines, ECM constituents and ECM degrading enzymes in NP cells. However, gene expression alterations may not be an accurate reflection of modulatory effects occurring at the protein level. Hence, whilst other cytokines and chemokines (IL-16, CCL2, CCL3, CCL7 and CXCL8) did not demonstrate transcriptional modulatory potential it is possible

that they may still exert translational modulatory potential that could affect protein expression.

6.3.3 Selection of Treatments for Protein Expression Studies

Cell culture supernatants collected from alginate cultures subjected to IL-1β stimulation were selected for further cytokine and chemokine protein expression analysis. These samples were selected on the basis of the modulatory potential of IL-1β on other cytokines and chemokines displayed at the level of gene expression described in this chapter.

6.3.4 Summary

Data presented in this chapter confirms the transcriptional modulatory potential of IL-1β on cytokines, chemokines, ECM components and ECM degrading enzymes in NP cells. Further, it demonstrated that for certain targets, particularly the ECM degrading enzymes, this modulation by IL-1β is dependent on NP cell phenotype. This investigation also demonstrates that IL-16, CCL2, CCL3, CCL7 and CXCL8 do not modulate cytokine, chemokine, ECM component or ECM degrading enzyme expression at the level of transcription in NP cells.

7 The Effect of Interleukin-1β Stimulation on Cytokine and Chemokine Release from Primary Human Nucleus Pulposus Cells *In Vitro*

7.1 Introduction

This chapter describes investigations into the modulatory effect of IL-1β on cytokine and chemokine release from primary NP alginate cultures. Multiplex bead immunoassays were used to simultaneously quantify 20 cytokines and chemokines in conditioned media from cultures under basal conditions and following stimulation with IL-1β.

7.1.1 Methods

Full details of methods used in this study are given in Chapter 2, sections;

- 2.4 Cell culture
- 2.6 Luminex Multiplex Bead Immunoassay

7.1.2 Specific Objective

This chapter aims to address the hypothesis that:

Regulatory inter-relationships exist between IL-1β and the cytokines and chemokines of the IVD.

The specific objective is:

- To determine the regulatory potential of IL-1β on cytokine and chemokine release from primary NP alginate cultures

7.2 Results

Expression of all cytokines and chemokines investigated was detected in conditioned media from alginate NP cultures under basal conditions (Figure 7-1). The cytokine expression profile was dominated by IL-6 (>100pg/mL), followed by CSF2 and TNF-α (>10pg/mL), IL-4 (~10pg/mL) and IL-1β (~5pg/mL). Low concentrations of IFN-γ, IL-10, IL-2 and IL-5 (<1pg/mL) were also detected. The chemokine expression profile was dominated by CXCL8 (>9,000pg/mL) and CCL2 (>2,000pg/mL), followed by CCL7 (~15pg/mL), CCL3, CXCL1 and CXCL9 (~7pg/mL), CCL4 (~4pg/mL), CCL5 (~2pg/mL) and CCL8 (~1pg/mL). Low concentrations of CCL11 and CXCL10 (<1pg/mL) were also detectable.

7.2.1 Cytokine Release Following IL-1β Stimulation

7.2.1.1 IL-1β Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-1β into culture media (Figure 7-2A). Increased release of IL-1β was observed separately in cells derived from all 5 IVDs investigated (data not shown). Stimulation ≥10pg/mL resulted in significant increase in IL-1β release from alginate cultures compared to basal levels (P<0.05).

7.2.1.2 IL-2 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-2 into culture media (Figure 7-2B). Increased release of IL-2 was observed separately in cells derived from 4 IVDs, standard curve generation failure prevented IL-2 investigation in cells derived from a fifth IVD (data not shown). Although a trend of increasing IL-2 release following stimulation was observed, this was only significant following 100ng/mL treatment (P=0.002).

7.2.1.3 IL-4 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-4 into culture media (Figure 7-2C). Increased release of IL-4 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in IL-4 release occurred following stimulation with ≥10pg/mL (P<0.05).

7.2.1.4 IL-5 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-5 into culture media (Figure 7-2D). Increased release of IL-5 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in IL-5 release occurred following stimulation with ≥10pg/mL (P<0.05).

7.2.1.5 IL-6 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-6 into culture media (Figure 7-2E). Increased release of IL-6 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in IL-6 release occurred following stimulation with ≥1pg/mL (P<0.05).

7.2.1.6 IL-10 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IL-10 into culture media (Figure 7-2F). Increased release of IL-10 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in IL-10 release occurred following stimulation with ≥10pg/mL (P<0.05).

7.2.1.7 TNF-α Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of TNF-α into culture media (Figure 7-3A). Increased release of TNF-α was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in TNF-α release occurred following stimulation with ≥10pg/mL (P<0.05).

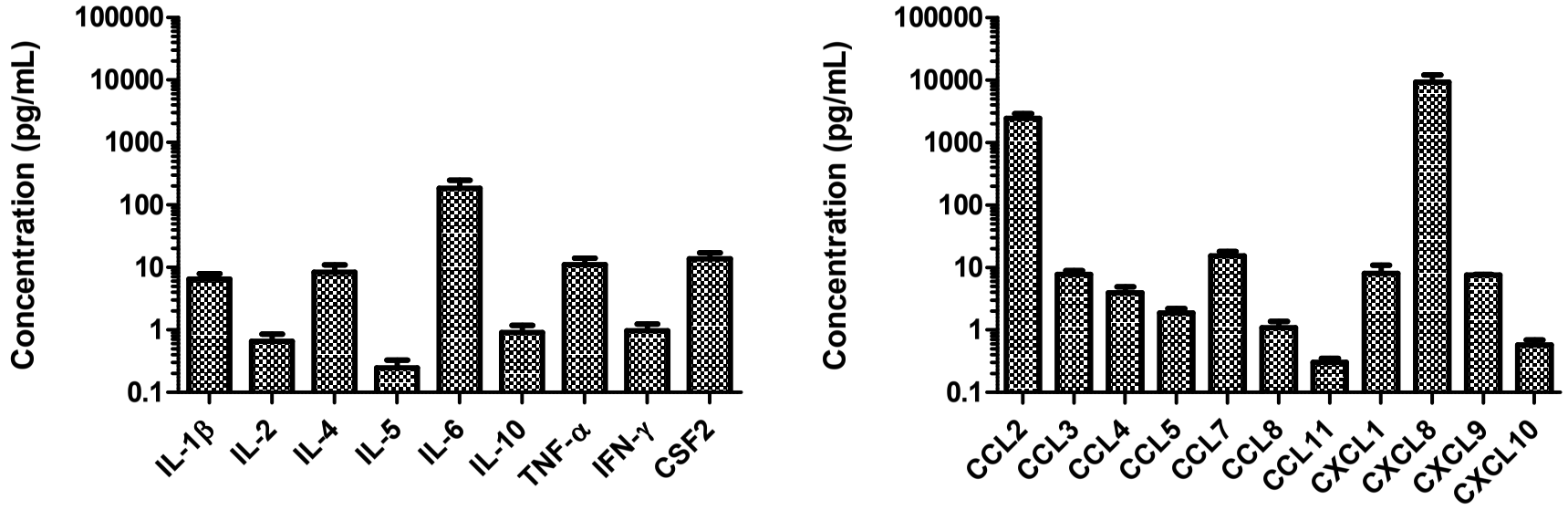


Figure 7-1 Basal Cytokine and Chemokine Release Profile of Alginate Cultured Primary NP Cells

Cytokines and chemokines in conditioned media following 48 hour incubation with alginate cultured primary human NP cells was quantified by Luminex multiplex bead immunoassay. Graphical data represents mean cytokine and chemokine concentration (± SEM). Data shown is obtained from primary alginate cultures derived from 5 NP tissue samples, each performed in triplicate, with the exceptions of IL-2, CSF2, CCL3, CCL8 and CXCL10 measurements that are obtained from primary alginate cultures derived from 4 NP tissue samples, and CXCL9 measurement that is obtained from primary alginate cultures derived from 2 NP tissue samples.

7.2.1.8 IFN-γ Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of IFN-γ into culture media (Figure 7-3B). Increased release of IFN-γ was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in IFN-γ release occurred following stimulation with ≥1pg/mL (P<0.05).

7.2.1.9 CSF2 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CSF2 into culture media (Figure 7-3C). Increased release of CSF2 was observed separately in cells derived from 4 IVDs, standard curve generation failure prevented investigation in cells derived from a fifth IVD (data not shown). Significant increase in CSF2 release occurred following ≥10pg/mL (P<0.05).

7.2.2 Chemokine Release Following IL-1β Stimulation

7.2.2.1 CCL2 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL2 into culture media (Figure 7-4A). Increased release of CCL2 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CCL2 release occurred following stimulation with ≥1pg/mL (P<0.05).

7.2.2.2 CCL3 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL3 into culture media (Figure 7-4B). Increased release of CCL3 was observed separately in cells derived from 4 IVDs, standard curve generation failure prevented investigation in cells derived from a fifth IVD (data not shown). Significant increase in CCL3 release occurred following stimulation with ≥10pg/mL (P<0.05).

7.2.2.3 CCL4 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL4 into culture media (Figure 7-4C). Increased release of CCL4 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CCL4 release occurred following stimulation with 100pg/mL (P<0.05).

7.2.2.4 CCL5 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL5 into culture media (Figure 7-4D). Increased release of CCL5 was observed separately in cells derived from all 5 IVDs investigated (data not shown).

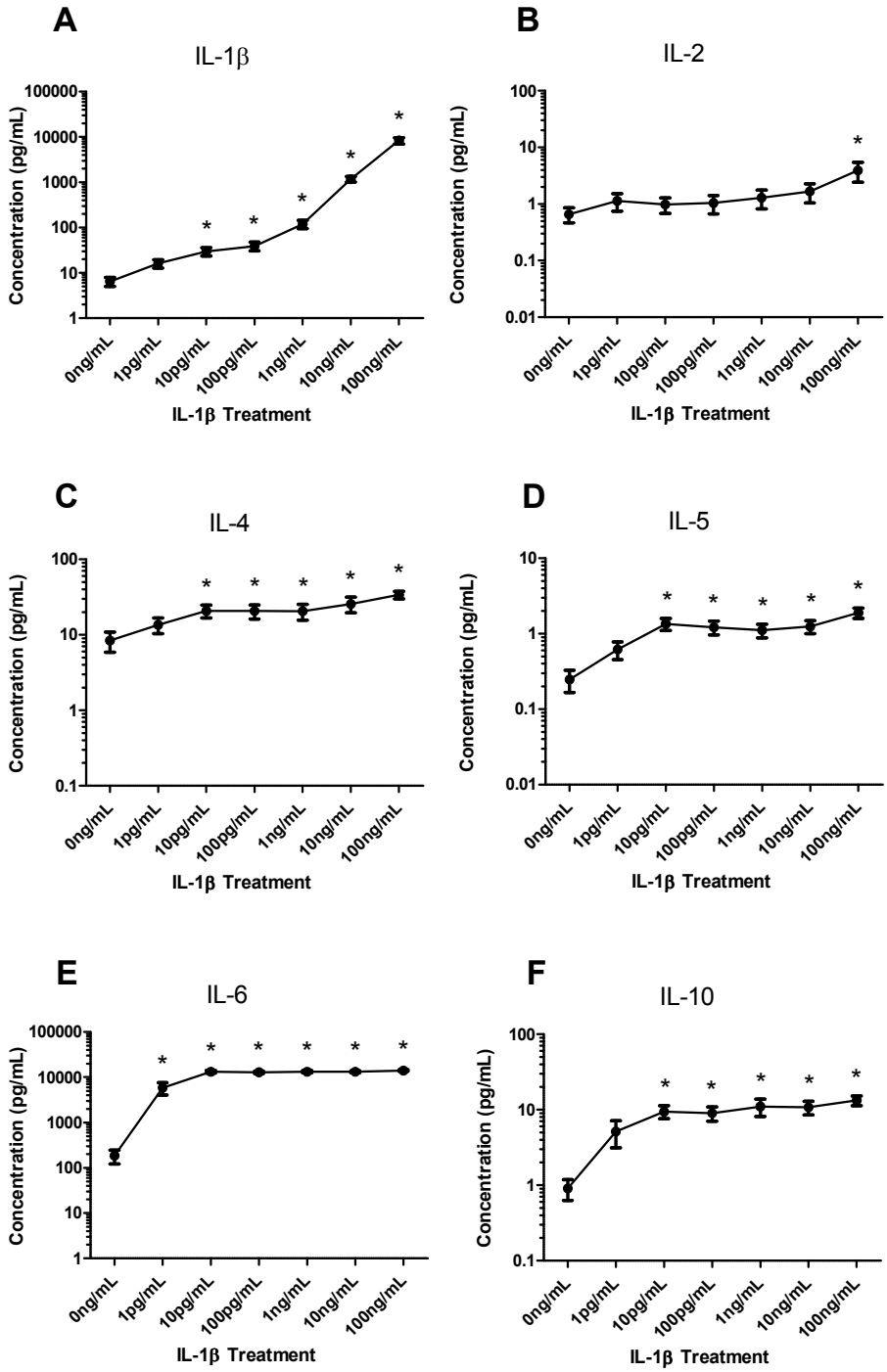


Figure 7-2 Interleukin Release from Alginate Nucleus Pulposus Cultures Following IL-1β Stimulation

Alginate cultured NP cells were subjected to 48 hour treatment with 0pg/mL, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β. Each treatment was performed in triplicate on cultures derived from five NP tissue samples, with the exception of IL-2 that was performed in triplicate on cultures derived from four NP tissue samples. Graphical data represents mean detection level ± SEM. * indicates $P<0.05$.

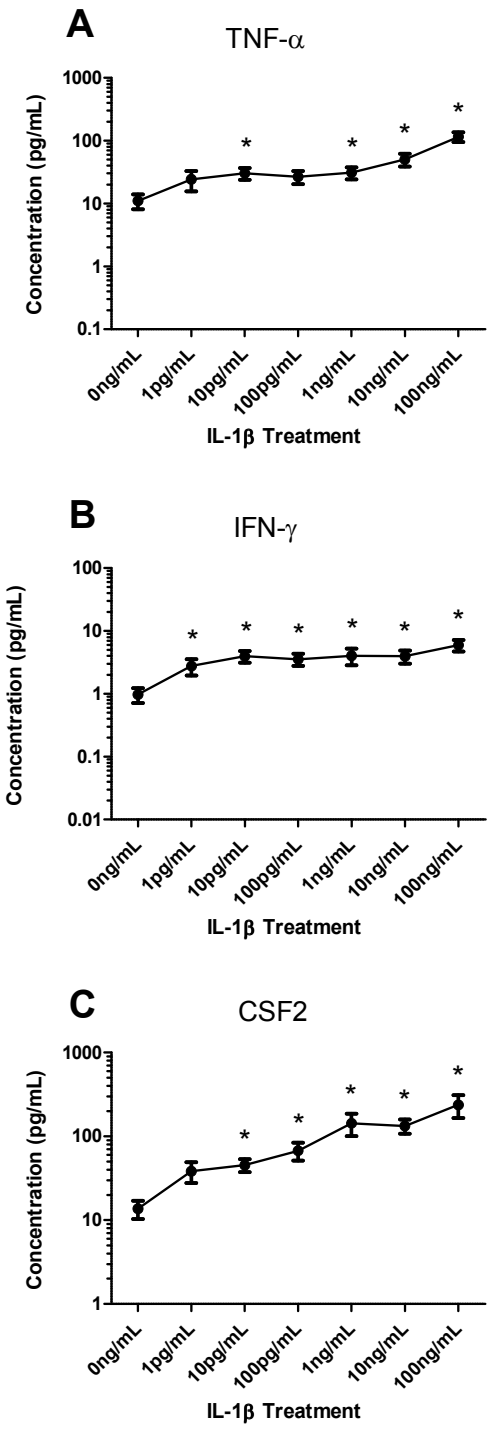


Figure 7-3 TNF-α, IFN-γ and CSF2 Release from Alginate Nucleus Pulposus Cultures Following IL-1β Stimulation

Alginate cultured NP cells were subjected to 48 hour treatment with 0pg/mL, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β. Each treatment was performed in triplicate on cultures derived from five NP tissue samples, with the exception of CSF2 that was performed in triplicate on cultures derived from four NP tissue samples. Graphical data represents mean detection level ± SEM. * indicates $P<0.05$.

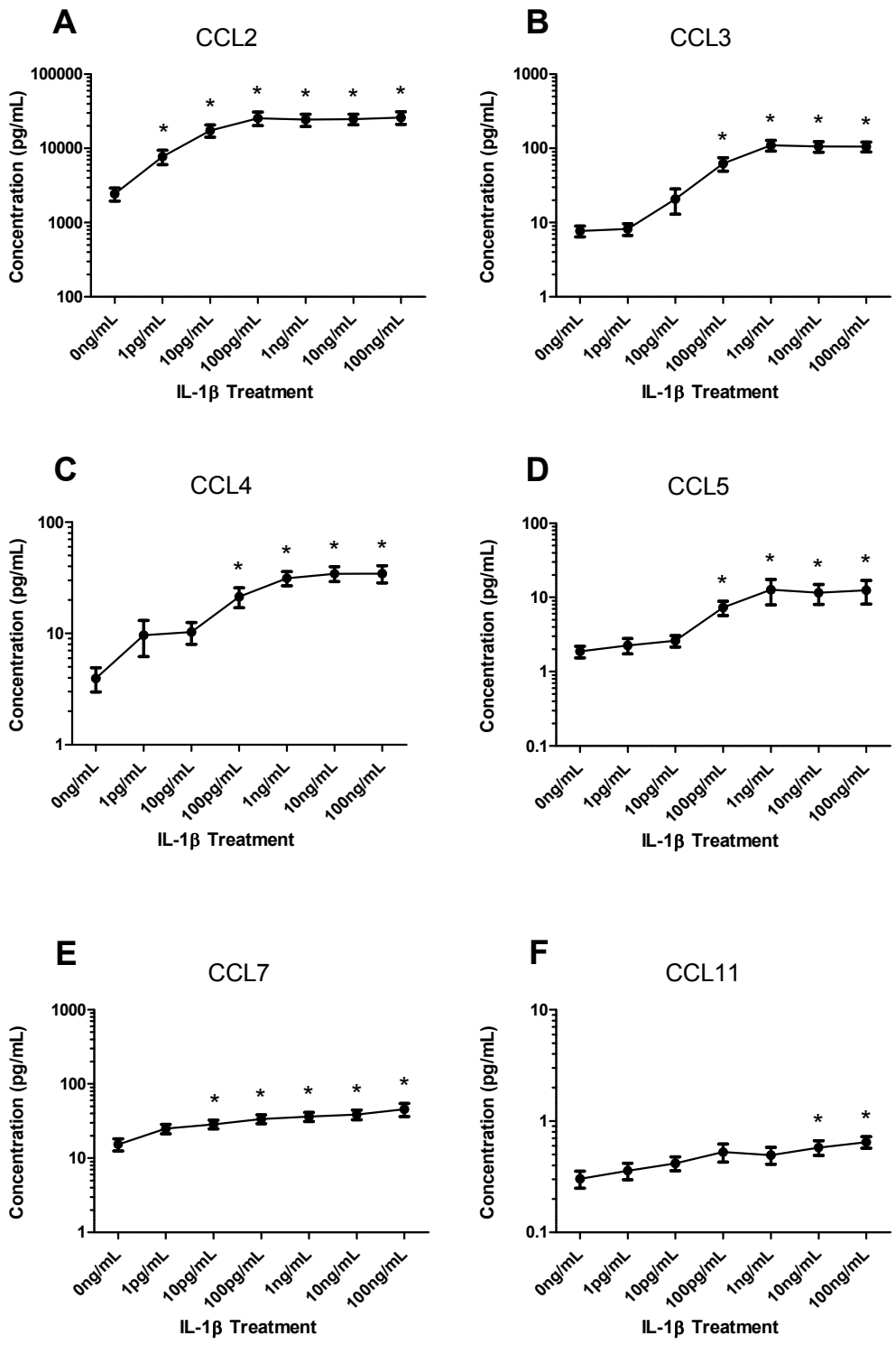


Figure 7-4 C-C Chemokine Release from Alginate Nucleus Pulposus Cultures Following IL-1β Stimulation

Alginate cultured NP cells were subjected to 48 hour treatment with 0pg/mL, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β. Each treatment was performed in triplicate on cultures derived from five NP tissue samples, with the exceptions of CCL3 and CCL8 that were performed in triplicate on cultures derived from four NP tissue samples. Graphical data represents mean detection level ± SEM. * indicates $P<0.05$.

Significant increase in CCL5 release occurred following stimulation with ≥100pg/mL (P<0.05).

7.2.2.5 CCL7 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL7 into culture media (Figure 7-4E). Increased release of CCL7 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CCL7 release occurred following stimulation with ≥10pg/mL (P<0.05).

7.2.2.6 CCL8 Release

IL-1β treatment did not affect CCL8 expression in alginate cultured NP cells. No effect of treatment was observed in cells derived from 4 IVDs, standard curve generation failure prevented investigation in cells derived from a fifth IVD (data not shown).

7.2.2.7 CCL11 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CCL11 into culture media (Figure 7-4F). Increased release of CCL11 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CCL11 release occurred following stimulation with ≥10ng/mL (P<0.05).

7.2.2.8 CXCL1 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CXCL1 into culture media (Figure 7-5A). Increased release of CXCL1 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CXCL1 release occurred following stimulation with ≥1pg/mL (P<0.05).

7.2.2.9 CXCL8 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CXCL8 into culture media (Figure 7-5B). Increased release of CXCL8 was observed separately in cells derived from all 5 IVDs investigated (data not shown). Significant increase in CXCL8 release occurred following stimulation with ≥1pg/mL (P<0.05).

7.2.2.10 CXCL9 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CXCL9 into culture media (Figure 7-5C). Increased release of CXCL9 was observed separately in cells derived from 2 IVDs, standard curve generation failure prevented investigation in cells derived from three other IVDs (data not shown). Although a trend of CXCL9 up-regulation was observed, this was only

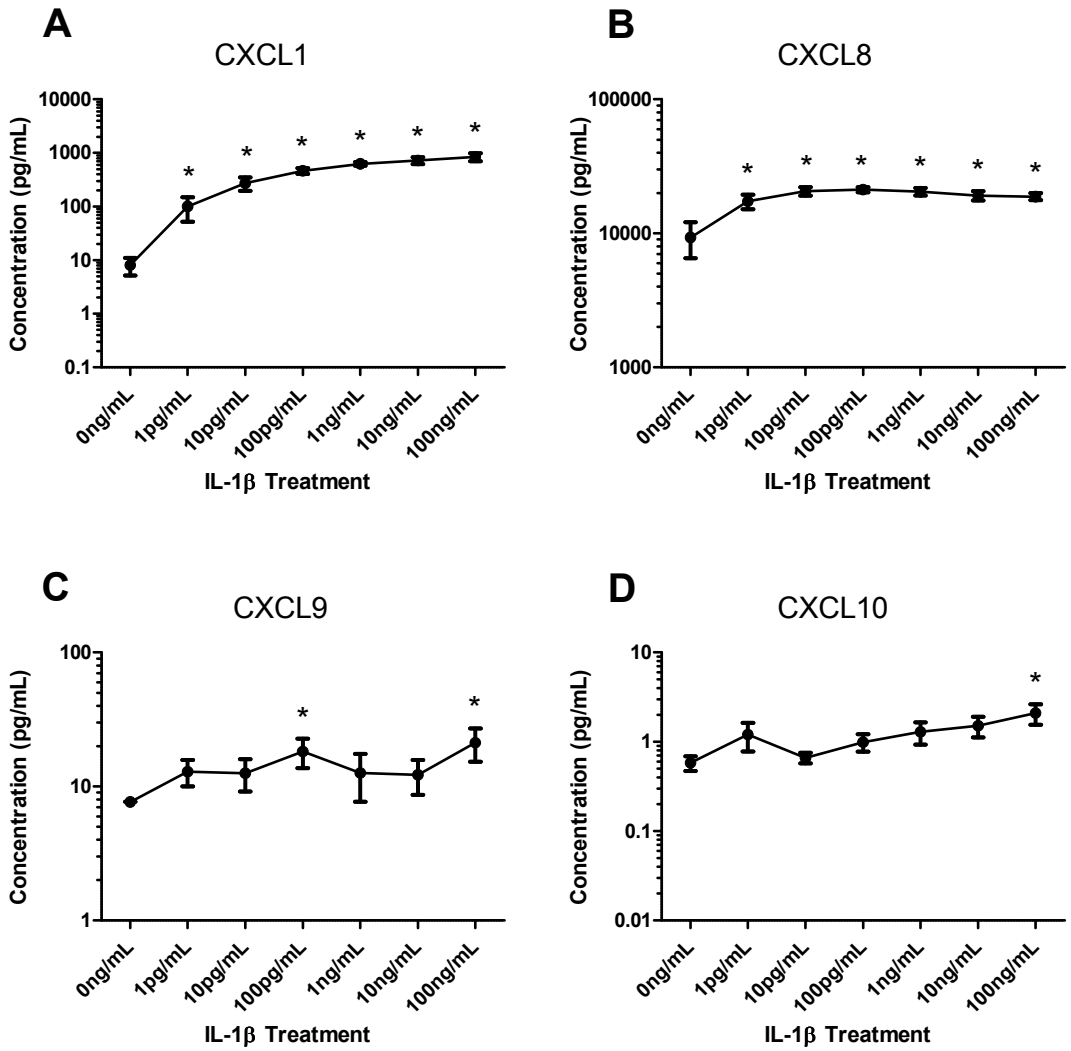


Figure 7-5 C-X-C Chemokine Release from Alginate NP Cultures Following IL-1β Stimulation

Alginate cultured NP cells were subjected to 48 hour treatment with 0pg/mL, 1pg/mL, 10pg/mL, 100pg/mL, 1ng/mL, 10ng/mL or 100ng/mL IL-1β. Each treatment was performed in triplicate on cultures derived from five NP tissue samples, with the exceptions of CXCL9 that was performed in triplicate on cultures derived from two NP tissue samples and CXCL10 that was performed in triplicate on cultures derived from four NP samples. Graphical data represents mean detection level ± SEM. * indicates $P<0.05$.

significant following 100pg/mL and 100ng/mL treatment (*P*=0.0225 and *P*=0.0151 respectively).

7.2.2.11 CXCL10 Release

Alginate cultured NP cells responded to 48 hour IL-1β treatment by increasing release of CXCL10 into culture media (Figure 7-5D). Increased release of CXCL10 was observed separately in cells derived from 4 IVDs, standard curve generation failure prevented investigation in cells derived from a fifth IVD (data not shown). Although a trend of increasing CXCL10 release following stimulation was observed, this was only significant following 100ng/mL treatment (*P*=0.0072).

7.2.3 Summary of Results

The results presented in this chapter are summarised in Table 7-1.

Target	IL-1β treatment effect	Target	IL-1β treatment effect
Cytokines		Chemokines	
IL-1β	↑*	CCL2	↑*
IL-2	↑*	CCL3	↑*
IL-4	↑*	CCL4	↑*
IL-5	↑*	CCL5	↑*
IL-6	↑*	CCL7	↑*
IL-10	↑*	CCL8	No effect
TNF-α	↑*	CCL11	↑*
IFN-γ	↑*	CXCL1	↑*
CSF2	↑*	CXCL8	↑*
		CXCL9	↑*
		CXCL10	↑*

Table 7-1 Summary of IL-1β Stimulation Effects on Cytokine and Chemokine Release from Alginate Cultured Nucleus Pulposus Cells

↑, increased release into culture media following IL-1β stimulation; *, significant increase in protein release compared to basal levels (*P*<0.05)

7.3 Discussion

The objective of this investigation was to determine the effects of IL-1β stimulation on modulation of cytokine and chemokine production by primary alginate NP cultures. It is worth noting at this point that the parameter measured in these investigations is cytokine and chemokine release into culture media. The quantifications made do not account for cytokines or chemokines synthesised but not released or those sequestered through GAG interactions with either the cell surface or ECM laid down within the alginate beads (Proudfoot, 2006).

Luminex multiplex bead immunoassays were used to interrogate conditioned media recovered from primary alginate NP cultures, by this method the presence and concentration of target analytes was determined. All cytokine targets investigated were detected in conditioned media from alginate cultures under basal conditions, indicating a diverse cytokine release profile is a feature of primary NP cells. Data generated indicates the cytokine expression profile is dominated by IL-6 (>100pg/mL), CSF2 (>10pg/mL) and TNF-α (>10pg/mL), all of which were detected previously at the mRNA level in our gene expression studies (see Chapter 3, section 3.2 and Chapter 4, section 4.2). The chemokine expression profile was dominated by CCL2 (>2,000pg/mL) and CXCL8 (>9,000pg/mL), both of which were detected previously at the mRNA level in our gene expression studies on NP cells isolated directly from NP tissue (see Chapter 3, section 3.2 and Chapter 4, section 4.2). NP cell protein production was also identified previously in tissue sections, (Chapter 5, section 5.2). CCL2 and CXCL8 gene expression was investigated in our previous study using alginate NP cultures, where constitutive expression under basal conditions was identified (see Chapter 6, section 6.2).

With the exception of CCL8, IL-1β stimulation significantly increased release of all cytokines and chemokines investigated from alginate NP cultures. Several different modulatory patterns were observed and these appeared to be IL-1β concentration dependent.

The targets that dominated the cytokine and chemokine protein expression profile under basal conditions; IL-6, CCL2 and CXCL8, were particularly sensitive to IL-1β stimulation. Significant increases in protein release occurred for IL-6 (32-fold), CCL2 (3-fold) and CXCL8 (2-fold) following stimulation with only 1pg/mL IL-1β. Other targets sensitive to this level of IL-1β stimulation were IFN-γ and CXCL1, where 3-fold and 13-fold increases in protein release were observed. Considering these five targets, three different modulatory responses were observed. For CXCL8 and IFN-γ, the increase in protein release following 1pg/mL stimulation was maximal - no further significant increase in expression was observed with increasing dose stimulation. For IL-6 and CCL2, a further significant increase in protein release, on top of that observed following 1pg/mL stimulation, was observed following stimulation with 10pg/mL, before maximal release was achieved. CXCL1 release increased in a dose dependent manner with significant increases between stimulation concentrations up to 1ng/mL prior to reaching maximal.

Increasing IL-1β stimulation to 10pg/mL was seen to modulate other cytokines and chemokines. Significant auto-induction of IL-1β (4.5-fold) occurred, alongside increases in protein release for IL-4 (2.5-fold), IL-5 (5.5-fold), IL-10 (10-fold), TNF-α (3-fold), CSF2 (3-fold) and CCL7 (2-fold). Considering these seven targets, three different modulatory responses were observed. For IL-1β and CSF2, the increase in protein release following 10pg/mL stimulation was maintained following stimulation with 100pg/mL however, protein release increased significantly again following stimulation with 1, 10 and 100ng/mL. For TNF-α, the protein release stimulated by 10pg/mL IL-1β was maintained following stimulation with 100pg/mL and 1ng/mL however, 10 and 100ng/mL stimulation resulted in further increases in protein release. For IL-4, IL-5, IL-10 and CCL7 the increase in protein release following 10pg/mL IL-1β stimulation was maximal – no further significant increase in expression was observed with increasing dose stimulation.

Increasing IL-1β stimulation to 100pg/mL extended the modulatory effect to other chemokines. Significant increases in protein release following 100pg/mL stimulation were detected for CCL3 (8.5-fold), CCL4 (5.5-fold) and CCL5 (4-fold). For CCL3, protein release was further significantly increased following 1ng/mL treatment before maximal levels were reached. For CCL4 and CCL5 the protein release stimulated by 100pg/mL IL-1β was maximal.

The four remaining targets; IL-2, CCL11, CXCL9 and CXCL10, were less sensitive to modulation by IL-1β. Stimulation with 10ng/mL was required before release of CCL11 was significantly increased compared to basal levels and stimulation with 100ng/mL was required to significantly increase release of IL-2 and CXCL10. As a result of standard curve generation failure CXCL9 release was only quantified in alginate cultures derived from two IVD samples. Data generated indicates a trend of increasing CXCL9 release following IL-1β stimulation although release was only significantly increased following 100pg/mL and 100ng/mL stimulation.

IL-1β stimulated release of IL-6, CCL2, CXCL8 and TNF-α from primary NP cultures has been investigated previously (Yoshida *et al.*, 2002; Huang *et al.*, 2008; Studer *et al.*, 2011). The effect of IL-1β stimulation on IL-6 release from primary NP cultures has been investigated in monolayer and alginate experimental systems (Huang *et al.*, 2008; Studer *et al.*, 2011). Monolayer cultured primary human NP cells derived from prolapsed IVDs responded to 24 hour, 30ng/mL IL-1β stimulation with an ~8.1 fold increase in IL-6 release into culture media measured by ELISA (Huang *et al.*, 2008). Alginate cultured primary human NP cells derived from

degenerate IVDs responded to 24 hour 0.5ng/mL IL-1β stimulation a ~611 fold increase in IL-6 release into culture media measured by ELISA (Studer *et al.*, 2011). Data generated in this investigation confirms these previous reports of IL-1β stimulated release of IL-6 from primary NP cultures and further, demonstrates that IL-6 release is not a dose dependent response to IL-1β stimulation.

The effect of IL-1β stimulation on CCL2 release from primary NP cultures has been investigated previously (Yoshida *et al.*, 2002; Huang *et al.*, 2008). These studies demonstrate CCL2 release from monolayer cultured primary human NP cells is increased ~3.2 fold following 24 hour 30ng/mL IL-1β stimulation (Huang *et al.*, 2008), here maximal CCL2 release (~10 fold) occurred following 10pg/mL IL-1β stimulation. The increased fold-release observed here may represent a differential response to IL-1β stimulation between monolayer and alginate cultured NP cells. Dose-dependence of CCL2 release following IL-1β stimulation has been investigated previously in monolayer cultured rabbit NP cells (Yoshida *et al.*, 2002). Treatment concentrations between 0.01 and 100ng/mL IL-1β were applied for 24 hours prior to quantification of CCL2 in culture media. Stimulation with 0.1ng/mL IL-1β was required to significantly increase CCL2 release (Yoshida *et al.*, 2002). Data generated here confirms previous reports of CCL2 release from NP cultures following IL-1β stimulation (Yoshida *et al.*, 2002; Huang *et al.*, 2008).

The effect of IL-1β stimulation on CXCL8 and TNF-α release from primary NP cultures has been investigated previously (Huang *et al.*, 2008). These studies demonstrate monolayer cultured primary human NP cells derived from prolapsed IVDs respond to 24 hour, 30ng/mL IL-1β stimulation by increasing release of CXCL8 (~2.3 fold) and TNF-α (~3 fold) into culture media, measured by ELISA (Huang *et al.*, 2008). The fold change in CXCL8 release reported in monolayer cultures (Huang *et al.*, 2008) is similar to the maximal 2-fold increase seen here in alginate NP cultures although we report a greater increase in TNF-α release (~10 fold), which may be related to a differential response between monolayer and alginate cultures.

IL-1β stimulated release of other cytokines and chemokines has not been investigated previously in NP cultures although some targets have been investigated in articular chondrocyte cultures or cartilage explants (Recklies & Golds, 1992; Villiger *et al.*, 1992; Pulsatelli *et al.*, 1999; Moos *et al.*, 2001). These studies demonstrate IL-1β stimulated release of CCL2 (Villiger *et al.*, 1992; Pulsatelli *et al.*, 1999), CCL3 (Pulsatelli *et al.*, 1999), CCL5 (Pulsatelli *et al.*, 1999),

CXCL1 (Recklies & Golds, 1992) and CXCL8 (Recklies & Golds, 1992; Pulsatelli *et al.*, 1999) from primary human articular chondrocytes derived from OA, RA and normal tissue donor cartilage samples. Stimulation of OA cartilage explant cultures also demonstrates the modulatory effect of IL-1β on chondrocyte production of IL-4 and IL-10, where an increase in the percentage of immune-positive cells is observed following IL-1β stimulation (Moo *et al.*, 2001).

7.3.1 Summary

Data generated in this investigation confirms the production of cytokines and chemokines by primary NP cells, and their release into the extracellular environment. The finding that produced cytokines and chemokines are secreted by NP cells confirms their potential to act as intercellular signals in paracrine signalling mechanisms or as autologus regulators in autocrine signalling mechanisms.

This investigation confirms the modulatory potential of IL-1β on cytokine and chemokine production at the protein level, as observed previously in gene expression studies (Chapter 6). Although it remains unclear from this investigation whether IL-1β stimulation affects protein synthesis or modulates the release of pre-formed intracellular protein stores.

The modulatory effects of IL-1β stimulation appeared to be dose-dependent with respect to the different cytokine and chemokine targets. Increasing the dose stimulation was seen to extend the modulatory influence of IL-1β to further cytokine and chemokine targets unresponsive to lower dose stimulation. This may indicate that IL-1β modulates cytokine and chemokine release by several different mechanisms, and that these mechanisms are induced in a concentration dependent manner. The observed patterns of cytokine or chemokine release may represent groups of cytokine and chemokine targets that are modulated by the same mechanism.

8 General Discussion

The overall aim of studies described in this thesis was to investigate the hypothesis that *cytokines and chemokines are integral to the pathogenesis of IVD degeneration and prolapse*, both of which may lead to the generation of LBP.

The first specific objective of these studies was to identify cytokines and chemokines produced within the NP of normal, degenerate and prolapsed IVDs, and the second, to identify the 'target cells' of these cytokines and chemokines based on receptor expression. These two objectives were addressed by the studies described in Chapters 3, 4 and 5 of this thesis, where human NP tissue was utilised to identify cytokines and chemokines produced by cells present within NP tissue samples, to identify the cellular source of this production, and to relate production to characteristic features of the pathology of IVD degeneration.

Previous studies have identified that cytokine and chemokine expression is increased in degenerate and prolapsed IVD tissue compared to that obtained from normal tissue donors (Burke *et al.*, 2002; Le Maitre *et al.*, 2005; Bachmeier *et al.*, 2007; Le Maitre *et al.*, 2007d). Based on these findings, it would appear that there is a direct association between cytokine and chemokine expression and IVD pathology, although whether this is cause or effect remains unknown.

The progression of IVD degeneration is known to proceed by the altered behaviour of native cells (Urban *et al.*, 2000), the pathological changes this causes to IVD tissue structures is known to increase the susceptibility of IVDs to prolapse injury (Adams & Hutton, 1985). Due to the association between pathology and cytokine expression a number of studies have attempted to understand the role that cytokines may play in the disruption of native cell behaviour. These studies have identified that the cytokines, IL-1 and TNF-α, can dysregulate ECM turnover (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d; Hoyland *et al.*, 2008; Millward-Sadler *et al.*, 2009; Studer *et al.*, 2011), by down-regulating rates of anabolic metabolism and up-regulating rates of catabolic.

With the exception of IL-1 and TNF-α, studies investigating the production of cytokines and chemokines within the IVD are limited. Initial observations of cytokine and chemokine expression in the IVD by Ahn *et al.* (2002) and Kawaguchi *et al.* (2002) identified a further 11 cytokine and chemokine targets however, to date detailed investigations into the expression or potential roles of these targets within the IVD have only been performed for IL-6 and IFN-γ (Shamji *et al.*, 2010; Gabr *et al.*, 2011; Studer *et al.*, 2011).

Chapters 3 and 4 of this thesis describe our initial investigations to determine the cytokine and chemokine gene expression profile of cells isolated directly from NP tissue. In total, 25 cytokine and 18 chemokine targets were investigated, alongside associated receptors and where relevant, activating enzymes and signalling accessory proteins. Of the 43 cytokine and chemokine targets investigated, gene expression of 35 was confirmed, 17 of which are novel findings to the IVD. Further, in 34 of the 35 cases, corresponding signalling receptor expression was also detected, indicating that the cytokines and chemokines produced in the IVD may function within it, as intercellular signalling molecules.

Chapter 4 describes a more detailed investigation into the gene expression profile of cells isolated directly from NP tissue samples. This study not only confirms gene expression of cytokines, chemokines and signalling receptors within the IVD, but identifies targets with differential expression profiles based on the pathology of tissue samples from which they were derived. The differential expression profile observed for certain targets indicates an association between target and pathology, although these investigations are not sufficient to indicate whether the relationship may be cause or effect.

An important observation was made during the course of studies described in Chapters 3 and 4, and this has implications both for the interpretation of data generated here, and for the design of future investigations into IVD pathology. That is, degenerative changes were not consistent throughout tissue samples, but rather present as 'degenerative lesions' surrounded by histologically normal tissue. The implications of this observation on data generated in these studies is that, it is impossible to know what percentage of cells isolated from any tissue sample are derived from a degenerate lesion or a histologically normal tissue region.

To overcome this limitation in future studies, it may be better to use the method of Laser Capture Microdissection (LCM) to isolate NP cells, rather than enzymatic digestion of ECM for cell isolation as used here. LCM uses sections of fixed tissue that are examined microscopically and cells 'captured' from their native position within the tissue section by laser dissection (Simone *et al.*, 1998). By this method, it would even be possible to perform a spatial comparison of target expression, if cells were collected separately from degenerate lesions and tissue appearing histologically normal within the same sample.

Studies using the method of LCM would address a further limitation of the investigations described in Chapters 3 and 4. That is, that the isolation of cells from tissue samples by enzymatic digestion of ECM constitutes an *in vitro*

manipulation, which may affect the cytokine and chemokine gene expression profile of isolated cells. Practically, because of the acellularity of NP tissue, cell isolation prior to RNA extraction is required to concentrate sufficient cell numbers into an appropriate reaction volume. However, mRNA expression, particularly of inducible targets, is often transient and stability is low, for example IL-16 mRNA half-life is only 2 hours (Cruikshank *et al.*, 2000). Hence, the time frame of enzymatic digestion (4 – 18 hours) is sufficient for mRNA expression profile alterations to occur.

Gene expression profiling generates large data sets that can be 'mined' to elucidate information related to specific parameters of interest. Data generated in Chapters 3 and 4 was interrogated in respect of relative levels of mRNA expression and the frequency at which detection was made, within particular study groups. It may be useful to subject this data to further analysis, related to the co-expression of targets investigated. In OA, two distinct cytokine phenotypes have been described; TNF-α^{High} characterised by TNF-α and IL-6 expression, and TNF-α^{Low} characterised by IL-1β, transforming growth factor-β, IL-4 and IL-10 expression (Moos *et al.*, 2000; Moos *et al.*, 2001). These distinct phenotypes are thought to arise from polymorphisms affecting the expression of IL-1β or IL-1Ra, and therefore link cytokine expression with a genetic predisposition in OA (Moos *et al.*, 2000). Further, co-expression can be an indication of molecules that share regulatory pathways, are modulated by the same stimuli, or exert modulatory potential over each other. It may be the case that cytokine co-expression patterns are characteristic of IVD pathology, and principle component analysis or multivariate analysis of data generated in these investigations may be sufficient to provide initial indications as to cytokine and chemokine co-expression patterns within the IVD.

Chapter 5 describes investigations by IHC into protein production and localisation of cytokines, chemokines and receptors. These studies were necessary in order to confirm that observed gene expression was translated into protein production within the IVD. For all targets investigated, protein expression by native NP cells was confirmed. Analysis of the expression patterns of these produced proteins allowed for the identification of differential expression profiles based on the pathology of the IVD. However, the differential protein expression profiles observed did not in all cases agree with the previously obtained gene expression profiles for the same targets across the same study groups.

Differences between mRNA and protein expression profiles arise primarily through two mechanisms, either mRNA production is transient, and produced transcripts

unstable, whilst the proteins produced have greater stability and increased half-life, remaining detectable over a longer time period (Villiger *et al.*, 1992), or translational modifications exert effects on rates of protein production that are not related to or detectable at, the level of mRNA transcription. Either scenario may account for the differences observed in these investigations, although since production is a prerequisite for activity, the protein expression profile is a better indication of factors likely to be active within the IVD.

The findings reported in Chapters 3, 4 and 5 of this thesis suggest that cytokine and chemokine expression is increased in the IVD in line with increasing severity of pathological tissue changes. Together, these studies add further evidence to the association between cytokine and chemokine expression and IVD pathology.

The third specific objective was to determine the inter-relationships that exist between the cytokines and chemokines of the IVD. This objective was addressed by studies described in Chapters 6 and 7 of this thesis, where *in vitro* manipulations of primary NP cultures were performed to determine the effect of cytokine or chemokine stimulation on NP cell behaviour.

Chapter 6 describes investigations to determine the modulatory effects of selected cytokines and chemokines on mRNA expression of target cytokines and chemokines. In total, the effects of stimulation with 6 different cytokines and chemokines were investigated, although only IL-1β was seen to modulate transcription of other cytokines and chemokines under the experimental conditions investigated.

Whilst no significant effects were observed for the other 5 cytokines and chemokines, this lack of modulatory potential cannot be considered conclusive. In a previous study by Villiger *et al*. (1992), the modulatory potential of IL-1β and LIF on mRNA expression of CCL2 was investigated. This study demonstrated that whilst both cytokines up-regulate CCL2 mRNA transcription, the kinetics of the response to IL-1β and LIF are different. Time course investigations indicated that LIF stimulation induced a rapid increase in CCL2 transcription that was maximal 2 hours post stimulation, and returned to basal levels by 5 hours post stimulation. The initial response to IL-1β stimulation was slower, taking 8 hours to reach maximal however, the effect was longer lasting (>11 hours). Both cytokines were seen to up-regulate levels of CCL2 detectable in conditioned media collected 24 hours post stimulation (Villiger *et al.*, 1992), and so modulate protein expression in a similar manner, but through different transcriptional modulatory mechanisms.

These investigations by Villiger *et al.* (1992) demonstrate that the kinetics of transcriptional response to cytokine stimulation is different depending on the cytokine applied. A limitation therefore, of investigations described in Chapter 6 of this thesis is that all stimulations were performed for 48 hours, the time point previously identified as optimal for detection of IL-1β stimulated responses (Le Maitre, 2003). As such, in order to confirm the transcriptional modulatory potential of the other cytokines and chemokines investigated, further time course investigations are required.

Chapter 7 describes investigations by immunoassay into production and secretion of cytokines and chemokines by alginate NP cultures following IL-1β stimulation. These studies were necessary in order to confirm that the transcriptional modulatory potential of IL-1β extended to the level of protein expression. In total, the effect of IL-1β stimulation on secretion of 20 cytokines and chemokines was investigated. For 19 of these targets, it was confirmed that IL-1β up-regulates secretion, although it is difficult to interpret the data relating to up-regulation of IL-1β since exogenous IL-1β was supplemented to the cultures.

The final objective of this thesis was to understand how the different cytokine and chemokine expression profiles and inter-relationships may contribute to the disruption of catabolic and anabolic cellular metabolism. This objective was addressed by studies described in Chapters 6 and 7, whereby the effects of cytokine stimulation on anabolic and catabolic metabolism were investigated alongside the quantification of cytokine and chemokine production by primary NP cultures, with and without IL-1β stimulation.

In a recent study by Liebscher *et al*. (2011) that investigated the cell population of the IVD, the young adult human lumbar NP was determined to contain ~2,600 cells/mm^3. This physiological value described by Leibscher *et al.* (2011) is slightly increased compared to the cell density used in investigations described in Chapters 6 and 7, where cell density equivalent to 2,000 cells/mm^3 was used. Accounting for the increased cell numbers *in vivo* the concentration of cytokines and chemokines within the IVD can be calculated, assuming that the culture model used accurately represents the *in vivo* scenario.

On account of this, basal IL-1β expression would be ~8.5pg/mL within the IVD. Considering the modulatory potential of IL-1β stimulation, as determined in Chapter 6, this concentration would stimulate ECM remodelling. Although the effect may not be detrimental, since gene expression of aggrecan was observed to be up-

regulated alongside the down-regulation of type II collagen, up-regulation of MMP-3 (stromelysin) and -13 (collagenase), whilst aggrecanolytic ADAMTS-4 transcription was not significantly affected. The net effect of this modulatory pattern may result in beneficial changes, promoting a more gelatinous and less collagenous ECM. This would indicate that the low level detection of IL-1β, identified previously in the normal IVD (Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2007d) may be beneficial to IVD structural integrity.

There has been some success in indentifying environmental conditions that can lead to IL-1 production within the IVD, such as excessive loading stresses (Wang *et al.*, 2007). The findings presented in this thesis suggest that this production of IL-1 in response to changing environmental conditions may be an appropriate response to trigger adaptive tissue remodelling. However, our findings also support the previously reported detrimental effects of excessive or increased IL-1β production on ECM structural integrity (Le Maitre *et al.*, 2005; Hoyland *et al.*, 2008; Millward-Sadler *et al.*, 2009; Studer *et al.*, 2011).

The findings reported in Chapters 6 and 7 of this thesis indicate that IL-1 is a key regulator of NP cell behaviour. IL-1β exerts significant modulatory potential on the production of other cytokines and chemokines by NP cells *in vitro*, and on transcription of genes related to ECM remodelling.

The context in which we sought to identify the cytokine and chemokine expression profile of the IVD was in so far as it related to the progression of IVD pathology, and to the effects that it may elicit on attempted biological therapies targeted at impeding progression of, or even reversing this pathology. In respect of IVD degeneration, pathological changes associated with altered cellular behaviour are related to proliferation (Johnson & Roberts, 2003), differentiation (Gruber *et al.*, 2005; Roberts *et al.*, 2006), dysregulated metabolism (Antoniou *et al.*, 1996a; Weiler *et al.*, 2002) and cell death (Park *et al.*, 2001a). It seems reasonable to assume that, if the cytokines and chemokines identified here do form part of the pathological mechanism of IVD degeneration and prolapse, their effects would be related to the adverse alterations in cell behaviour observed in that pathology.

Aside of the investigations described previously, the effects of cytokines and chemokines on NP cell behaviour have not been comprehensively studied, although their effects in other cell types have been described. Chondrocytes exhibit morphological and biochemical similarity to NP cells, and so the effects of cytokines and chemokines on chondrocyte behaviour may be relevant to that of NP cells. In chondrocytes, effects of cytokine and chemokine stimulation have been

described in relation to all of the common features of altered NP cell behaviour as seen in IVD degeneration. These include increased rates of proliferation (Mazzetti *et al.*, 2004), hypertrophic differentiation (Merz *et al.*, 2003; Wei *et al.*, 2010), increased catabolic metabolism (Carroll *et al.*, 1995; Alaaeddine *et al.*, 2001; Yuan *et al.*, 2001; Mazzetti *et al.*, 2004), decreased anabolic metabolism (Alaaeddine *et al.*, 2001; Yuan *et al.*, 2001), and induced cell death (Borzi *et al.*, 2002; Wei *et al.*, 2006). The precedents set in these investigations indicate that further investigative work into the role of cytokines and chemokines within the IVD is warranted.

Elucidation of the pathology of IVD degeneration is required for the successful development of treatment strategies. IVD degeneration increases the risk of LBP development (Luoma *et al.*, 2000) and 40% of LBP cases are linked directly to IVD degeneration (Schwarzer *et al.*, 1995). Successful treatment of IVD degeneration therefore represents a mechanism by which the incidence of LBP within the population may be decreased.

Currently, several research initiatives are investigating the regenerative potential of stem cell therapy within the IVD to enhance the degenerate cell population (Steck *et al.*, 2005; Le Maitre *et al.*, 2009; Collin *et al.*, 2011; Yang *et al.*, 2011). Application of the soluble factor, TGF-β, elicits well described effects on mesenchymal and adipose-derived stem cell differentiation, indeed its application *in vitro* is required to stimulate differentiation towards a NP phenotype (Steck *et al.*, 2005; Yang *et al.*, 2011). The survival and differentiation characteristics of stem cells injected into NP explant cultures, under conditions modelling the environment of the 'normal' IVD have been confirmed (Le Maitre *et al.*, 2009). However, the findings presented in this thesis suggest that cells implanted into the degenerate IVD would encounter a diverse and altered range of soluble factors, including cytokine and chemokine signalling molecules. The effects of these on stem cell behaviour have not been investigated, and clearly the response of implanted cells to their surrounding environment will have considerable influence over the success of stem cell therapy to the IVD. To this end, the studies described in this thesis provide insight into the conditions of the 'degenerate niche' and may form the basis of further investigations to determine the effects this environment may elicit on stem cell behaviour.

8.1 Future Work

Accumulating evidence supports a direct association between cytokine and chemokine expression and IVD pathology. The investigations described in this thesis have identified NP cells as a source of numerous cytokines and chemokines,

although the effects that these elicit in and around the IVD are largely unknown. Further investigation, to determine whether these factors affect NP cell behaviour in respect of proliferation, differentiation and cell death is warranted since this may link expression with pathology.

Although preliminary investigations into the effects of cytokines and chemokines in dysregulated NP cell metabolism were described in this thesis, the studies were by no means conclusive. Further work in this area, including; time course investigations into the effects of cytokine and chemokine stimulation on transcription of ECM components and ECM degrading enzymes, and investigations at the protein level into synthesis of ECM components and ECM degrading enzymes would help to clarify the regulatory role of cytokines and chemokines in tissue remodelling.

Whilst we have identified the production of chemokines and chemokine receptors within the IVD, the studies described here are not sufficient to confirm their biological activity. Biological activity of secreted chemokines is controlled at the levels of interaction with ECM GAGs (Proudfoot, 2006), and proteolytic processing (Wolf *et al.*, 2008). The conserved chemokine N-terminal domain can be exposed or protected by ECM GAG interaction following sequestration within the ECM, once exposed this domain becomes susceptible to proteolytic cleavage. Chemokine proteases included members of the MMP and ADAM families that are often present in the ECM milieu, proteolytic cleavage of chemokines can serve to activate, enhance, dampen or inactivate biologic activity and modulate the affinity of a chemokine to a specific receptor (Wolf *et al.*, 2008).

The activity of chemokine receptors is also subject to multiple levels of regulation. Functional activity requires receptors to be presented on the cell surface in a conformation compatible with ligand binding and coupling to a heterotrimeric G protein (Bennett *et al.*, 2011). Post-translational modifications including sulphation and glycosylation are known to influence receptor conformation and therefore compatibility with ligand binding (Farzan *et al.*, 1999; Sloane *et al.*, 2005), and phosphorylation affects receptor presentation or internalization modulating a cells capacity to respond to chemokine signalling (Horuk, 2001). Investigating the biological activity of chemokines and chemokine receptors in the IVD would provide knowledge as to the conditions under which they are active.

In respect of LBP, the finding that NP cells secrete cytokines and chemokines confers the potential for these to function as biochemical stimuli on local nociceptive nerve fibres. This may be particularly relevant to pain arising from IVD

prolapse since elevated cytokine and chemokine levels are found in the cerebrospinal fluid of IVD prolapse patients (Brisby *et al.*, 2002) and a recent investigation using an animal model of IVD prolapse indicates that pain intensity is associated with NP derived CXCL8 production (Kim *et al.*, 2011). Therefore, investigations into the effects of NP derived cytokines and chemokines on nerve cells may provide insight into the mechanisms by which LBP is generated.

8.2 Conclusions

The studies described in this thesis investigated in detail the expression of cytokines and chemokines by the NP cell population of the IVD. Evidence presented has shown that expression of certain cytokines and chemokines is up-regulated in NP cells from the degenerate IVD compared to those from the normal counterpart, and that expression correlates with pathological tissue changes characteristic of degeneration. Co-expression of receptors for these molecules was also identified, indicating that these factors may form part of an intercellular cytokine and chemokine communication network within the IVD.

In vitro, NP cells were shown to secrete a diverse range of cytokines and chemokines, and IL-1 was demonstrated to be a modulator of this activity. Indeed, data presented is further evidence to suggest IL-1 is a key modulator of NP cell behaviour. Here, IL-1 exerted modulatory potential over expression of other cytokines and chemokines, and modulated anabolic and catabolic metabolism. *In vitro* stimulations with other cytokines and chemokines were less conclusive as to the effects these may elicit on NP cell behaviour however, together the data presented implicates that cytokines and chemokines are integral to the pathogenesis of IVD degeneration and prolapse.

9 Appendices

Appendix 1 Human Intervertebral Disc Tissue Samples

Reference		Source	Sex	Age	IVD Level	IVD Intact?	Grade			qPCR Classification	Infiltrated?
HD	1	Surgical	M	42	L4/L5	No	4.0	4.2	3.5	D	Yes
HD	2	Surgical	F	40	L5/S1	Yes	7.3	5.7	4.5	D	Yes
HD	3	Surgical	M	25	L4/L5	Yes	4.0	4.7	5.7	D	No
HD	4	Surgical	F	48	L4/L5	Yes	4.0	5.7	4.7	D	Yes
HD	5	Surgical	F	33	L5/S1	Yes	9.0			D	No
HD	6	Surgical	-	-	-	-	6.0	7.0	4.2	D	Yes
HD	8	Surgical	F	62	L4/L5	Yes	7.5	7.3	7.5	D	No
HD	9	Surgical	M	32	L5/S1	Yes	2.3	3.0	2.9	N	No
HD	10	Surgical	M	26	L5/S1	Yes	3.3	2.3		N	No
HD	11	Surgical	F	53	L4/L5	No	7.5	7.5	3.5	D	Yes
HD	12	Surgical	F	40	L5/S1	Yes	7.4	5.5	6.2	D	No
HD	14	Surgical	F	66	L5/S1	-	5.7	5.5	4.5	D	Yes
HD	15	Surgical	F	34	L4/L5	Yes	6.2	7.5	9.2	D	No
HD	16	Surgical	M	49	L5/S1	Yes	7.0			D	No
HD	17	Surgical	M	45	L5/S1	Yes	4.0			D	No
HD	18	Surgical	M	35	L4/L5	No	4.0			D	Yes
HD	21	Surgical	F	26	L4/L5	Yes	4.0			D	Yes
HD	22	Surgical	M	23	L4/L5	Yes	2.7			N	No
HD	23	Surgical	F	29	L4/L5	Yes	7.8			D	Yes
HD	24	Surgical	M	35	L5/S1	No	3.0			N	No

Reference		Source	Sex	Age	IVD Level	IVD Intact?	Grade			qPCR Classification	Infiltrated?
HD	25	Surgical	M	20	L4/L5	No	3.0			N	Yes
HD	26	Surgical	F	39	L5/S1	No	4.5			D	Yes
HD	27	Surgical	M	73	L4/L5	Yes	4.7			D	No
HD	28	Surgical	M	69	L3/L4	Yes	6.5			D	No
HD	29	Surgical	M	37	L4/L5	Yes	6.9			D	No
HD	30	PM	M	45	L4/L5	Yes	1.8	2.0	2.0	N	No
HD	31	PM	M	45	L3/L4	Yes	3.0	2.5	3.5	N	No
HD	32	PM	M	45	L5/S1	Yes	3.5	5.0		D	No
HD	33	Surgical	M	48	L4/L5	No	9.5	6.0	8.4	D	No
HD	34	Surgical	F	26	L5/S1	No	11.0	3.5	3.9	D	No
HD	36	Surgical	F	33	L5/S1	Yes	8.5	6.5	7.4	D	Yes
HD	37	PM	F	74	L1/L2	Yes	10.0	6.0	5.5	D	No
HD	38	PM	F	74	L4/L5	Yes	8.0	6.0	8.5	D	No
HD	39	PM	F	74	L5/S1	Yes	7.0	9.0	8.0	D	No
HD	40	PM	F	74	L3/L4	Yes	8.0	7.3	8.5	D	No
HD	41	PM	F	74	L2/L3	Yes	11.0	10.3	9.0	D	No
HD	44	Surgical	M	42	L5/S1	Yes	3.0			N	No
HD	45	Surgical	F	36	L5/S1	Yes	8.0	4.0	5.9	D	No
HD	46	Surgical	F	41	L5/S1	Yes	7.5			D	No
HD	49	Surgical	M	-	-	-	6.0	4.7	5.0	D	Yes
HD	50	Surgical	M	44	L5/S1	Yes	3.0	2.3	4.4	N	Yes

Appendix 1 Continued from previous page.

Reference		Source	Sex	Age	IVD Level	IVD Intact?	Grade			qPCR Classification	Infiltrated?
HD	51	Surgical	F	52	L4/L5	Yes	10.5	7.0	5.4	D	No
HD	52	Surgical	M	-	L4/L5	No	9.0	6.2	4.0	D	Yes
HD	53	Surgical	F	38	L5/S1	No	5.8	5.2	4.5	D	Yes
HD	54	Surgical	F	28	L4/L5	Yes	7.7	3.5	7.4	D	No
HD	55	Surgical	-	-	L5/S1	No	8.5			D	Yes
HD	56	Surgical	F	43	L5/S1	No	5.8			D	No
HD	57	Surgical	F	44	L5/S1	Yes	6.3			D	No
HD	58	Surgical	F	28	L5/S1	No	6.0			D	No
HD	59	Surgical	M	35	L5/S1	Yes	7.0			D	No
HD	60	Surgical	F	38	L5/S1	Yes	6.9			D	Yes
HD	61	Surgical	F	43	L5/S1	No	5.5			D	No
HD	63	Surgical	F	42	L5/S1	No	9.3			D	No
HD	64	Surgical	M	46	L5/S1	-	5.4			D	Yes
HD	66	Surgical	M	62	L3/L4	Yes	5.5			D	Yes
HD	71	Surgical	M	41	L5/S1	Yes	4.2			D	Yes
HD	73	Surgical	M	35	L4/L5	-	8.0			D	No
HD	74	Surgical	M	-	L4/L5	No	4.0			D	No
HD	75	Surgical	M	40	L3/L4	Yes	7.4			D	No
HD	79	Surgical	F	45	L4/L5	No	2.6			N	Yes
HD	85	Surgical	M	47	L2/L3	No	5.4			D	Yes
HD	86	Surgical	M	39	L5/S1	Yes	3.9			N	Yes

Appendix 1 Continued from previous page.

Reference		Source	Sex	Age	IVD Level	IVD Intact?	Grade	qPCR Classification	Infiltrated?
HD	89	Surgical	F	19	L5/S1	No	5.2	D	No

PM, Post-mortem; M, Male; F, Female; N, Non-degenerate; D, Degenerate.

Appendix 1 Continued from previous page.

Appendix 2 Disc Material Details Form

DISC MATERIAL DETAILS

Disc samples for Dr Christine Le Maitre
Please phone **0114 225 6163** or **0783 350 2227** if a disc sample is available for collection – please keep sample in fridge until collected

Sex	
D.O.B	
Weight	
Height	

Date of operation		
Diagnosis		
Disc Level		
Reason for operation (tick appropriate boxes)	Spinal Pain	
	Root Pain	
	Trauma	
	Infection	
	Other please state	
Operation title		
Previous operations		
Medications		
Radiological features/ Thompson grade if available		
Any other significant features		

ADDITIONAL INFORMATION FOR PROTRUSIONS

Extent of protrusion	1: Sequestered disc fragments separate from disc	
	2: Extrusion of nucleus material from the annulus	
	3: Attenuated annulus but intact	
	4: Annular bulge	
Time since onset of radicular pain		

Appendix 3 Automated Tissue Processing Schedule

	Time (minutes)
50% IMS	90
70% IMS	60
99% IMS	60
99% IMS	60
99% IMS	60
99% IMS	90
99% IMS	90
Sub-X	90
Sub-X	90
Sub-X	90
Molten Wax	90
Molten Wax	90

Appendix 4 Low Density Array Assay Identification Numbers and Threshold Values

(I)	1	2	3	4	5	6	7	8	9	10	11	12
A	Hs99999901s1	Hs99999905m1	Hs99999909m1	Hs99999903m1	Hs99999906m1	Hs01064648m1	Hs00222035m1	Hs00166223m1	Hs01006727m1	Hs00171042m1	Hs00171149m1	Hs00266213s1
B	Hs00174092m1	Hs00174106m1	Hs00369400m1	Hs99999083m1	Hs01075667m1	Hs00914532m1	Hs00332759m1	Hs00194264m1	Hs00236937m1	Hs00234140m1	Hs00171125m1	Hs00152917m1
C	Hs00174097m1	Hs00189606m1	Hs00155517m1	Hs00174143m1	Hs00902334m1	Hs00262062m1	Hs00538167m1	Hs00384278m1	Hs00236966m1	Hs00234142m1	Hs00171086m1	Hs00171121m1
D	Hs00174122m1	Hs00174383m1	Hs00218888m1	Hs00171165m1	Hs00387004m1	Hs00296982m1	Hs01042313m1	Hs00158730m1	Hs00171061m1	Hs99999148m1	Hs00174146m1	Hs01013469m1
E	Hs00174131m1	Hs00975262m1	Hs00372324m1	Hs00171455m1	Hs00175123m1	Hs00977691m1	Hs00961755m1	Hs00174099m1	Hs00171085m1	Hs00174575m1	Hs00174304m1	Hs00174764m1
F	Hs00174202m1	Hs00171163m1	Hs00174128m1	Hs00991002m1	Hs00234415m1	Hs00977695m1	Hs00538896m1	Hs00370506m1	Hs00237017m1	Hs00171147m1	Hs00171041m1	Hs00365842m1
G	Hs00174086m1	Hs00370528m1	Hs00236874m1	Hs00174759m1	Hs00542604m1	Hs00205346m1	Hs00166144m1	Hs00169146m1	Hs00174103m1	Hs00271615m1	Hs00174298m1	Hs01011079s1
H	Hs00174148m1	Hs00224471m1	Hs00171266m1	Hs00166237m1	Hs00181217m1	Hs00376373m1	Hs01114427m1	Hs00234224m1	Hs00171065m1	Hs00234646m1	Hs00174150m1	Hs00174299m1

(II)	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	ACTB	PGK	IL-17RA	IL-22RαI	IFN-γRα	gp130	CXCL10	CCL19	CCR3
B	IL-1α	IL-15	IL-17F	CSF3	IL-6R	IL-17RB	IL-23R	IFN-γRβ	CXCL1	CCL2	CCL20	CCR5
C	IL-1β	IL-16	IL-18	IFN-γ	IL-7R	IL-17RC	IL-12Rβ1	OSMR	CXCL2	CCL3	CX3CL1	CCR6
D	IL-4	IL-17A	IL-20	OSM	IL-10Rα	IL-17RD	TNF-R55	LIFRα	CXCL3	CCL4	CXCR1	CCR7
E	IL-6	IL-17B	IL-23	LIF	IL-10Rβ	IL-18Rα	TNF-R75	IL-1Ra	CXCL5	CCL5	CXCR2	CCR8
F	IL-7	IL-17C	TNF-α	IL-1RI	IL-11Rα	IL-18Rβ	GM-CSFRα	IL-1RAcP	CXCL6	CCL7	CXCR3	CX3CR1
G	IL-10	IL-17D	TNF-β	IL-1RII	IL-15Rα	IL-20RI	GM-CSFRβ	ICE	CXCL8	CCL8	CCR1	Duffy
H	IL-11	IL-17E	CSF2	IL-4R	CD4	IL-20RII	G-CSFR	TACE	CXCL9	CCL13	CCR2	D6

(III)	1	2	3	4	5	6	7	8	9	10	11	12
A	0.75	0.5	0.5	0.4	0.5	0.5	0.2	0.5	0.5	0.2	0.2	0.2
B	0.2	0.2	0.2	0.2	0.5	0.5	0.2	0.5	0.2	0.15	0.2	0.2
C	0.25	0.25	0.17	0.2	0.2	0.5	0.2	0.5	0.5	0.4	0.25	0.2
D	0.2	0.2	0.25	0.2	0.25	0.25	0.5	0.2	0.2	0.2	0.2	0.2
E	0.25	0.2	0.2	0.2	0.5	0.25	0.2	0.2	0.5	0.2	0.2	0.2
F	0.2	0.2	0.15	0.5	0.15	0.2	0.2	0.25	0.2	0.5	0.25	0.2
G	0.2	0.5	0.25	0.2	0.5	0.15	0.2	0.2	0.25	0.2	0.2	0.2
H	0.25	0.2	0.2	0.5	0.4	0.15	0.2	0.5	0.2	0.2	0.2	0.25

LDA details (I) Assay ID, (II) Gene Symbol, (III) Threshold Settings

Appendix 5 Chemokine Systematic Nomenclature, Synonyms & Acronyms

Systematic Name	Other Names
CCL2	MCP-1 (Monocyte Chemoattractant Protein-1)
CCL3	MIP-1α (Macrophage Inflammatory Protein-1α)
CCL4	MIP-1β (Macrophage Inflammatory Protein-1β)
CCL5	RANTES (Regulated on Secretion Normal T-cell Expressed and Secreted)
CCL7	MCP-3 (Monocyte Chemoattractant Protein-3)
CCL8	MCP-2 (Monocyte Chemoattractant Protein-2)
CCL13	MCP-4 (Monocyte Chemoattractant Protein-4)
CCL19	ELC (Epstein-Barr Virus-Induced Receptor Ligand Chemokine)
CCL20	LARC/MIP-3α (Liver and Activation-Related Chemokine/Macrophage Inflammatory Protein-3α)
CXCL1	Gro-α (Growth Related Oncogene-α)
CXCL2	Gro-β (Growth Related Oncogene-β)
CXCL3	Gro-γ (Growth Related Oncogene-γ)
CXCL5	ENA-78 (Epithelial Cell-Derived Neutrophil-Activating Factor, 78 Amino Acids)
CXCL6	GCP-2 (Granulocyte Chemoattractant Protein-2)
CXCL8	IL-8 (Interleukin-8)
CXCL9	Mig (Monokine Induced by γ-Interferon)
CXCL10	γIP-10 (γ-Interferon-Inducible Protein-10)
CX3CL1	Fractalkine
CCR1	C-C Chemokine Receptor 1
CCR2	C-C Chemokine Receptor 2
CCR3	C-C Chemokine Receptor 3
CCR5	C-C Chemokine Receptor 5
CCR6	C-C Chemokine Receptor 7
CCR7	C-C Chemokine Receptor 8
CCR8	C-C Chemokine Receptor 8
CXCR1	C-X-C Chemokine Receptor 1
CXCR2	C-X-C Chemokine Receptor 2
CXCR3	C-X-C Chemokine Receptor 3
CX3CR1	C-X3-C Chemokine Receptor 1
Duffy	Duffy Blood Group Chemokine Receptor
D6	Chemokine Binding Protein 2

Appendix 6 Suppliers Details

Abcam	Cambridge, UK
Ambion	Paisley, UK
Applied Biosystems	Warrington, UK
Beckton Dickinson	Oxford, UK
Bioline	London, UK
Fisher Scientific	Loughborough, UK
Gibco	Paisley, UK
GraphPad Software Inc.	San Diego, USA
Invitrogen	Paisley, UK
JeioTech	Supplied by *Jencons*
Jencons	East Grinstead, UK
Leica	Milton Keynes, UK
Luminex Corporation	Austin, USA
MediaCybernetics	Marlow, UK
Nunc	Supplied by *Fisher Scientific*
Olympus Corporation	Tokyo, Japan
Peprotech	London, UK
Qiagen	Crawley, UK
Sanyo Electric Co. Ltd.	Osaka, Japan
Shandon Elliott	Supplied by *Jencons*
Sigma	Dorset, UK
StatsDirect Ltd.	Altrincham, UK
Vector Laboratories	Peterborough, UK

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